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Aus dem Institut für Physiologische Chemie

Cell type-dependent release of nitric oxide and/or reactive nitrogen-
oxide species from intracellular SIN-1. Effects on cellular NAD(P)H

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1 INTRODUCTION

1.1 *Nitric oxide*

For a long time nitric oxide (NO) has been known as a rather toxic gas that reacts with oxygen to yield the even more harmful NO₂ radical (Kirsch, M., et al. 2002). On the other hand, nitric oxide was identified as an important messenger molecule in living organisms (Furchgott, R. F. and Zawadzki, J. V. 1980), that was at first called endothelium derived relaxing factor (ERDF). The ERDF was discovered in 1980 (Furchgott, R. F. and Zawadzki, J. V. 1980) and in human beings in 1987 (Palmer, R. M. J., et al. 1987). Later on the ERDF was identified as nitric oxide because of its parallelism to nitrates, i.e. glyceroltrinitrate.

Three nitric oxide-generating isoenzymes (nitric oxide synthases NOS I-III) are known in human beings (Gabriel, C., et al. 1997, Gryglewski, R. G., et al. 2002). We differentiate between neuronal NOS, nNOS=NOS I, inducible NOS, iNOS=NOS II and the endothelium NOS, eNOS=NOS III (Gabriel, C., et al. 1997, Gryglewski, R. G., et al. 2002). In all cases the amino acid L-arginine is - in the presence of the cofactor tetrahydrobiopterin, the coenzyme NADPH, and oxygen - oxidized to citrulline thereby generating NO (Kwon, N. S., et al. 1989). Whereas the iNOS is induced in inflammation and shock (Gabriel, C., et al. 1997, Palmer, R. M. J., et al. 1987) the endothelial NOS is mainly activated by acetylcholine. In all cases of activation, the released nitric oxide activates the heme-containing enzyme soluble guanylyl cyclase (sGC) (Ahlnér, J., et al. 1991, Feelisch, M. and Noack, E.

1989, Schönafinger, K. 1999, Schrammel, A., et al. 1998) thus increasing the second messenger cGMP (cyclic guanosine monophosphate) (Gabriel, C., et al. 1997, Kankaanranta, H., et al. 1997).

Since it is known that NO is able to diffuse across cell membranes (Gabriel, C., et al. 1997) there is no general need that exogenous NO must be formed intracellularly.

A variety of physiological effects of NO are evident, e.g. vasodilatation by smooth muscle relaxation, regulation of neurotransmission, and immune defence (Kankaanranta, H., et al. 1997, Lohse, M. J., et al. 1998). Additionally, there are some reports, that NO directly inhibits the respiratory chain, most likely by inhibition of the cytochrome oxidase (Brown, G. C. 1999, Granger, D. L. and Lehninger, A. L. 1982, Schweizer, M. and Richter, C. 1994).

At low concentrations, NO produced by nNOS and eNOS, located in the endothelium and the brain, has beneficial effects like vasodilatation and anti-aggregation of platelets (Karow, T. and Lang-Roth, R. 2002, Palmer, R. M. J., et al. 1987). In inflammation or shock, the iNOS produces orders of magnitude more nitric oxide than the other nitric oxide synthases, and this may cause dangerous hypotension and cell damage (Karow, T. and Lang-Roth, R. 2002, Palmer, R. M. J., et al. 1987). Some authors reported about some protective effects of endogenous NO from nNOS, mediated by NMDA receptor modulation (Manzoni, O., et al. 1992) and it is believed that NO exert an antioxidative effect via scavenging of highly harmful radicals like the HO[•] radical (Dendorfer, A. 1996).

Healthy endothelial cells are able to release nitric oxide, that causes on the one hand relaxation of smooth muscle cells of vessels, mainly of veins and coronary arteries, and on the other hand anti-aggregation of platelets. In either arteriosclerosis or stenosis of a vessel, the body is not able to compensate the deficit of endogenous nitric oxide, and such diseases go together with a dysfunction of endothelial NO release (Dendorfer, A. 1996, Gryglewski, R. G., et al. 2002). Thus the application of an exogenous nitric oxide releasing drug is then the main therapeutic goal in order to compensate for the deficit of endogenous NO. Therefore, a variety of nitric oxide releasing drugs were developed during the past years for treatment of angina pectoris, ischaemic heart disease, chronic heart failure, heart insufficiency, atherosclerotic, and hypertensive diseases (Dendorfer, A. 1996, Feelisch, M. 1998, Wilson, I. D., et al. 1987).

The main group of therapeutic NO donors are organic nitrates, such as glyceroltrinitrate (GTN), isosorbitedinitrate (ISDN) and pentaerythrityltetranitrate (PETN), which generate mainly enzyme-dependently nitric oxide (Feelisch, M. 1998). Another group of NO donors, including molsidomine, only generate NO in a non-enzymatic way.

1.2 The sydnonimine Molsidomine

Molsidomine, commonly named as Corvaton®, is a pharmacoon rapidly absorbed by the gastro-intestinal tract (Singlas, E. and Martre, H. 1983). Then it is enzymatically hydrolyzed by hepatic esterases (Schrammel, A., et al. 1998) to yield CO₂, ethanol, and SIN-1 (Schrammel, A., et al. 1998). Figure 1 shows the decomposition of molsidomine releasing NO and O₂⁻. Consequently, Molsidomine

is a so-called prodrug (Feelisch, M. and Noack, E. 1989), decomposing into the active metabolite SIN-1.

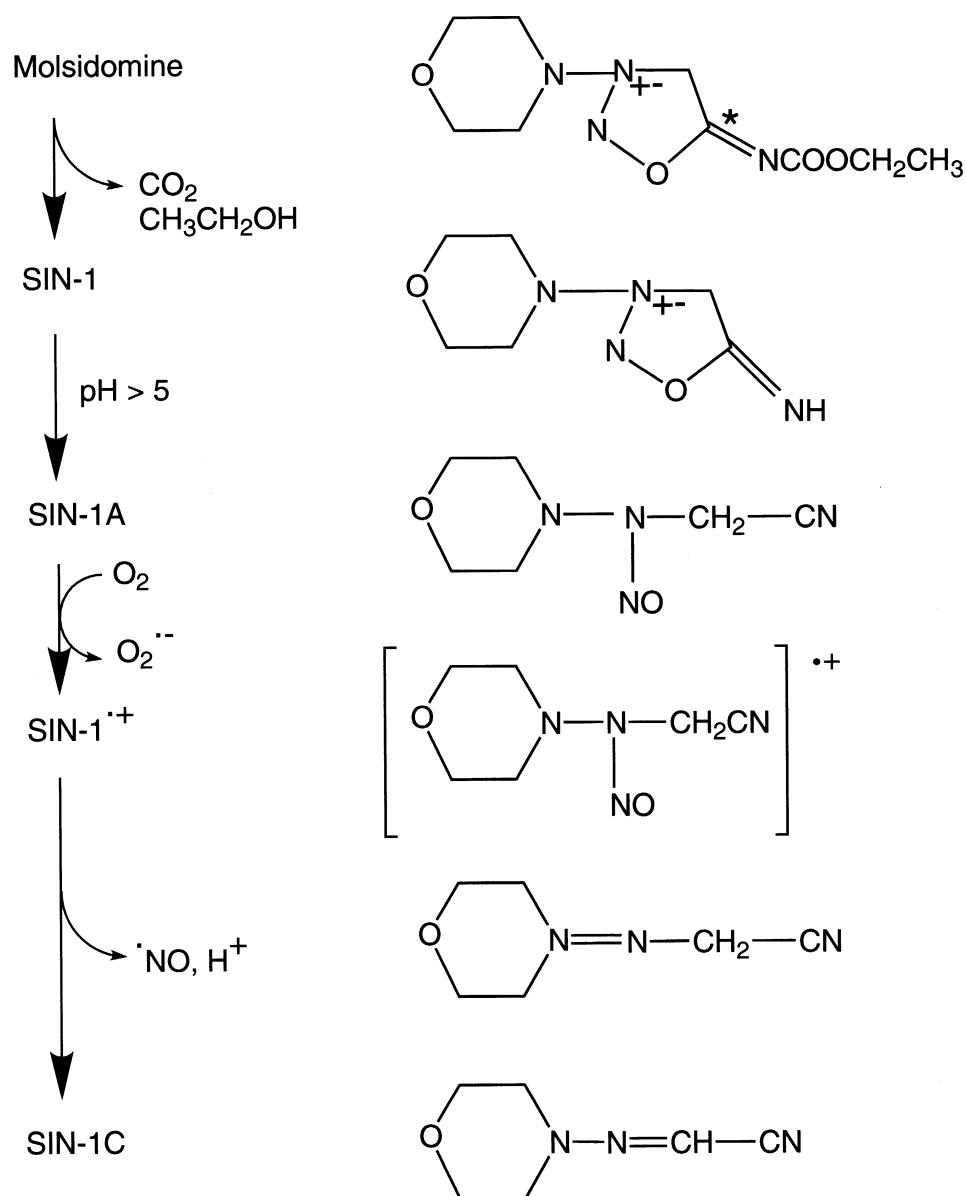


Figure 1: Mechanism of NO and $\text{O}_2^{\bullet-}$ release from Molsidomine in *in vitro* systems: Molsidomine enzymatically decomposes to SIN-1 thus releasing CO_2 and ethanol. At a $\text{pH} > 5$ it spontaneously decomposes to SIN-1A. In the presence of O_2 SIN-1 $^{\bullet+}$ is yielded under the release of $\text{O}_2^{\bullet-}$. Finally, splitting yielded NO, H^+ and the pharmacologically inactive form SIN-1C.

1.3 SIN-1

SIN-1 is the abbreviation for 3-Morpholinosydnonimine. In 1970 in Sydney it was first synthesized (Matusa, K. and Imashiro, Y. 1970) and such chemical functions are called sydnonimines. The half-time of this pharmacological entity lies between 60 and 90 minutes.

SIN-1 decomposes spontaneously to the substance SIN-1A (Fig.1) (Schönafinger, K. 1999). The pharmacological efficacy of SIN-1 is believed to be mediated by this substance, SIN-1A, which in the presence of O_2 spontaneously fragmentizes to yield SIN-1C, nitric oxide, and superoxide, respectively (Schönafinger, K. 1999) (Fig.1).

In some literature the decay of SIN-1 is explained via the intermediate $SIN-1^{\cdot+}$, a radical cation, and $O_2^{\cdot-}$ which are directly produced from SIN-1A (Chun-Qi, L. 2002). The conversion from SIN-1A to $SIN-1^{\cdot+}$ and SIN-1C, respectively, proceeds spontaneously and cannot be inhibited nor counteracted (Ashai, Y., et al. 1971, Wilson, I. D., et al. 1987). SIN-1C is known as the inactive endproduct of this reaction.

1.4 SIN-1C

SIN-1C will be oxidized in the liver to three components called metabolite E (*N*-Cyanomethylamino-*N*-(2'-hydroxyethyl)-glycine), metabolite E_1 (*N*-cyanomethylamino-2-amino-ethoxy)-acetic acid) (Wilson, I. D., et al. 1986) and small amounts of the metabolite I (*N*-cyanomethyleneamino-diethanolamine). Further metabolism can take place in generating SCN^- , probably from

intermediarily cyanide ion which is formed from the nitrile-containing metabolites of molsidomine.

1.5 Influence of pH and oxygen on decomposition of SIN-1

Under strictly anaerobic conditions SIN-1A is stable at pH 7.4 in cell-free experimental systems, but other electron acceptors than oxygen are expected to stimulate the decay of SIN-1A. Oxygen consumption may become important when sydnonimines are applied to biological systems. Besides, superoxide and protons (H^+) are generated during the decay of SIN-1, which may lead to a considerable decrease in pH in unbuffered aqueous solutions (Schönafinger, K. 1999). There is a correlation of oxygen consumption and NO formation in buffered SIN-1 solutions (Feelisch, M., et al. 1989). It is known that increasing oxygen concentrations enhances nitric oxide generation from SIN-1.

1.6 Releasing compounds of SIN-1:

Superoxide and nitric oxide generate peroxynitrite

As mentioned before, SIN-1 spontaneously decomposes to SIN-1C, superoxide and nitric oxide. The latter two radicals - superoxide and nitric oxide - recombine in a diffusion-controlled reaction to the harmful compound peroxynitrite $ONOO^-$ ($k = 3.9 \cdot 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Kissner, R. and Koppenol, W. H. 2002, Ross, A. B., et al. 1998)). Figure 2 shows 3 hepatocytes as representative cells in order to demonstrate the known chemical reactions (black arrow) and hypothetical cell membrane permeation pathway (green arrow) of SIN-1, SIN-1C and NO.

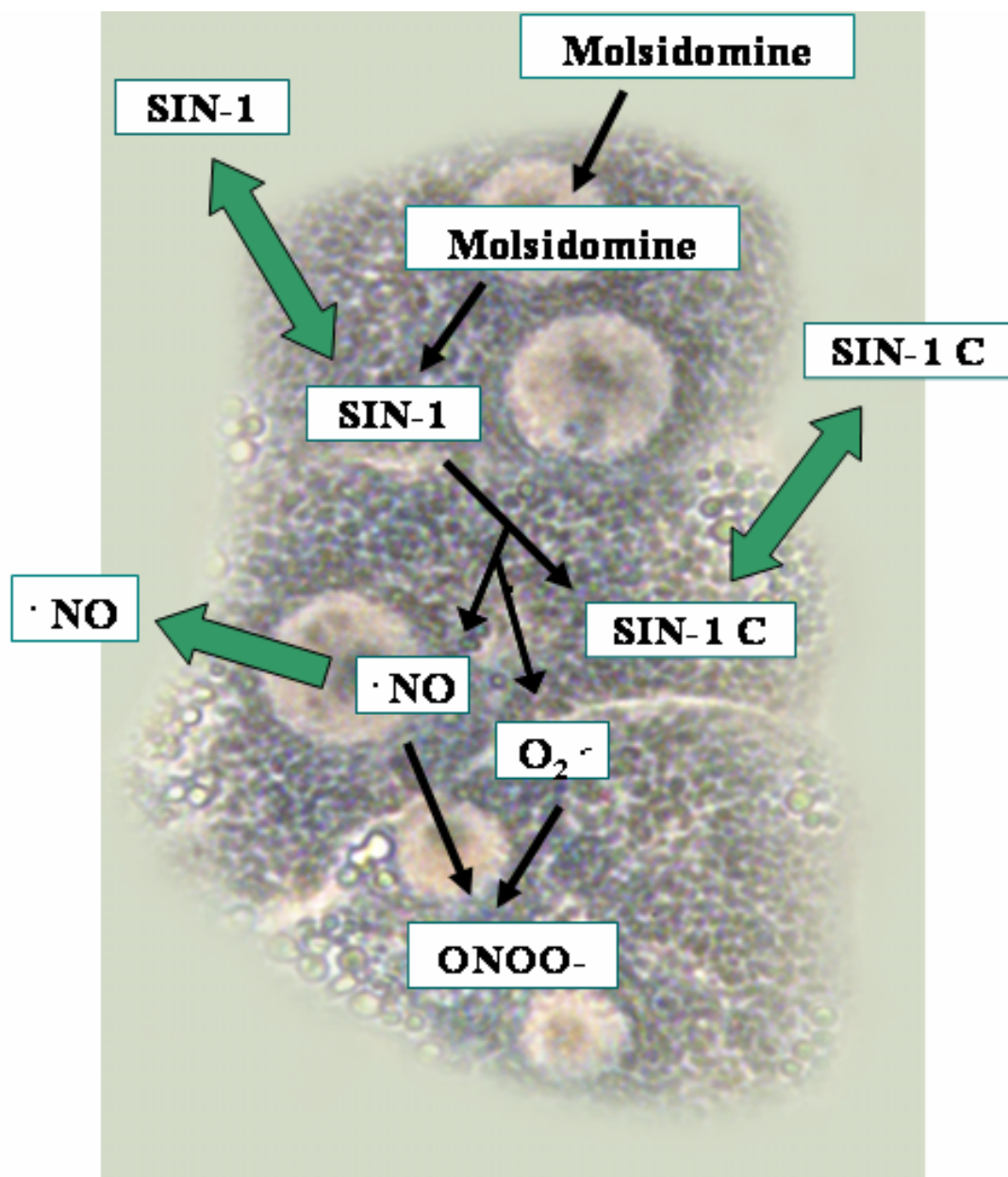


Figure 2: Intracellular pathway of Molsidomine in hepatocytes: 3 hepatocytes as representative cells demonstrate the known chemical reaction of SIN-1 to SIN-1C, $\text{O}_2^{\cdot-}$ and NO . These reactions are shown with black arrow. Hypothetical reactions are marked with green arrows. Molsidomine enters the hepatocytes and decomposes to SIN-1. SIN-1 might pass cell membranes or decompose inside to its decomposition product SIN-1C, thereby releasing nitric oxide and superoxide. The latter compounds may react to peroxynitrite. Also SIN-1C and nitric oxide may be able to pass the cell membrane, indicated by the green arrow.

1.7 Superoxide

As the superoxide dismutase activity is high in most cells (Huie, R. E. and Padmaja, S. 1993, Tyler, D. D. 1992) and because electron acceptors other than oxygen can additionally stimulate the decay of SIN-1A (Werringloer, J., et al. 1990), one can expect that nitric oxide rather than peroxynitrite is generated *in vivo* and that it is intracellularly generated from SIN-1, provided SIN-1 enters the cells. To the best of our knowledge, however, it is at present not known whether SIN-1 can penetrate cells. In experimental cell culture studies this highly important possibility is generally ignored because SIN-1 is frequently used as an extracellular peroxynitrite source, see e.g. (Bouton, C., et al. 1997, Chun-Qi, L. 2002, Doulias, P. T., et al. 2001, Garcia-Nogales, P., et al. 2003, Kaji, T., et al. 2002).

1.8 Aims of the study

Since SIN-1 is often used in experimental systems for the *in situ* generation of stoichiometric amounts of peroxynitrite (ONOO^-) (Feelisch, M., et al. 1989, Gryglewski, R. G., et al. 2002, Huie, R. E. and Padmaja, S. 1993) and regarding the fact that SIN-1 initially yields nitric oxide and superoxide it is actually unclear whether SIN-1 and its degradation product SIN-1C are capable to pass cell membranes thus generating nitric oxide and peroxynitrite intracellularly or only peroxynitrite extracellularly. In the present investigation L-929 mouse fibroblasts were selected as a typical tissue cell. Due to the fact that peroxynitrite and nitric oxide diametrically affected the cellular NAD(P)H-level, the following questions were clarified:

- 1.) Can SIN-1 and SIN-1C penetrate cell membranes?
- 2.) Does the cell type support the release of NO from SIN-1?
- 3.) Are reactive nitrogen-oxide species intracellularly formed?
- 4.) Is the content of NAD(P)H in the cytosol, mitochondrion and nuclei affected by SIN-1?

2 MATERIALS AND CELL CULTURE

2.1 Chemicals

Chemical	Company
Penicillin	Gibco (Eggenstein, Germany)
Streptomycin	Gibco (Eggenstein, Germany)
Potassium cyanide KCN	Behring (Mannheim, Germany)
Bovine serum albumin BSA	Behring (Mannheim, Germany)
Fetal bovine serum	Sigma (Steinheim, Germany)
Superoxide Dismutase SOD	Roche (Basel, Switzerland)
Minimum essential medium Eagle MEM	Sigma (Steinheim, Germany)
Metaphosphoric acid (8%)	Sigma (Steinheim, Germany)
Tri-Glycine	Sigma (Steinheim, Germany)
L-Glutamine	Sigma (Steinheim, Germany)
Pluronic	Sigma (Steinheim, Germany)
Probenecid	Sigma (Steinheim, Germany)
Spermine NONOate	Situs (Düsseldorf, Germany)
Dimethyl Sulfoxide DMSO	Merck (Darmstadt, Germany)
<u>Fluorescent dyes:</u>	
Fluorescent Nitric Oxide Cheletropic Trap	Prof. Dr. Dr. R. Sustmann
F-NOCT-1AM and FNOCT-1	Department of Organic Chemistry, Essen University
Diaminofluorescein-diacetate DAF-2 DA	Alexis Biochemicals, (Grünberg, Germany)

SIN-1 and SIN-1C	Drs. K. Schönafinger and J. Pünter (Aventis, Frankfurt/ Main, Germany)
Gases (5%CO ₂ /21%O ₂ /74%N ₂)	Messer-Griessheim (Oberhausen, Germany)
Cell culture plastic flasks 175cm ²	Falcon (Heidelberg, Germany)
Cell culture plastic flasks 75 cm ²	Falcon (Heidelberg, Germany)
Cell culture six-well-plastic flasks	Falcon (Heidelberg, Germany)
Cell culture plastic plates	Falcon (Heidelberg, Germany)
Glass coverslips	Assistent (Sondheim/ Röhn, Germany)

All chemicals were of the highest purity commercially available.

2.2 Buffer solutions

<i>PBS (phosphate buffered saline):</i>	pH 7.4
NaCl	136.0 mM
KCl	2.7 mM
Na ₂ HPO ₄	10.2 mM
KH ₂ PO ₄	1.5 mM
<i>MOPS (3-morpholino-propane-sulfone acid) buffer</i>	pH 6.3
NaCl	137.0 mM
KCl	5.4 mM
CaCl ₂	1.0 mM
MgCl ₂	0.5 mM

KH_2PO_4	0.4 mM
--------------------------	--------

MgSO_4	0.4 mM
-----------------	--------

Na_2HPO_4	0.3 mM
---------------------------	--------

Daily added:

L-Glutamine	2.0 mM
-------------	--------

MOPS (3-morpholino-propane-sulfone acid)	2.5 mM
--	--------

Buffer A	pH 7.4
-----------------	--------

NaCl	136.0 mM
------	----------

KCl	5.4 mM
-----	--------

CaCl_2	1.3 mM
-----------------	--------

MgCl_2	0.4 mM
-----------------	--------

KH_2PO_4	0.4 mM
--------------------------	--------

MgSO_4	0.7 mM
-----------------	--------

Na_2HPO_4	0.4 mM
---------------------------	--------

NaHCO_3	23.0 mM
------------------	---------

Pluronic	0.1 %
----------	-------

Probenecid	1.0 mM
------------	--------

Daily added:

Tri-glycine	25.0 mM
-------------	---------

L-Glutamine	2.0 mM
-------------	--------

Hanks balanced salt solution (modified):	pH 7.4
---	--------

NaCl	137.0 mM
------	----------

KCl	5.4 mM
-----	--------

CaCl ₂	1.0 mM
MgCl ₂	0.5 mM
KH ₂ PO ₄	0.4 mM
MgSO ₄	0.4 mM
Na ₂ HPO ₄	0.3 mM
<u>Daily added:</u>	
L-Glutamine	2.0 mM
Tri-glycine	25.0 mM

2.3 Cell culture of L-929 mouse fibroblasts

The murine fibroblast-derived cell line L-929 was obtained from the American Type Cell Culture Collection (ATTC, NCTC clone 929 of strain L). Cells were cultured in minimum essential medium Eagle, MEM, supplemented with 25 mM sodium bicarbonate, 10% fetal calf serum, 2 mM L-glutamine, penicillin (50 units/ml) and streptomycin (50 µg/ml) in 75 cm² plastic flasks at 37°C in a humidified atmosphere of 5%CO₂/21%O₂/74%N₂. Subcultivation was performed by trypsinisation (removing cells from each other and their culture plates using trypsin) every 2-3 days (Lomonosova, E., et al. 1998).

For experiments using capillary zone electrophoresis 5.0 x 10⁶ cells were seeded onto 176 cm² plastic flasks and cultured for 48 h (final density: 2.0 x 10⁷ cells).

For fluorescence and nitric oxide measurements 1.0 x 10⁵ cells were seeded onto 6.2 cm² glass coverslips in 6-well cell culture plates and cultured for 48 hours. Experiments with L-929 mouse fibroblasts were started on day 2 after subcultivation.

For the experiments performed with the nitric oxide electrode, the digital fluorescence microscope and the laser scanning microscope, a buffer solution (buffer A, pH 7.4, ingredients see above) was prepared.

3 METHODS

3.1 *Determination of the uptake of SIN-1 and SIN-1C in L-929*

mouse fibroblasts

The cellular uptake of SIN-1 and its stable decomposition product SIN-1C, respectively, were determined using capillary zone electrophoresis (Beckman P/ACE 5000; Beckman, Munich, Germany). SIN-1 100-fold stock solutions were prepared with phosphate buffer (50 mM KH_2PO_4 , pH 5.0) at 4°C and kept on ice until use (within 30 minutes). 48 h before starting experiments L-929 mouse fibroblasts were cultured in MEM on 176 cm² plastic flasks. On the day of the experiment, cells were washed three times with phosphate buffered saline (PBS, ingredients see above) and then incubated in a humidified atmosphere (5%CO₂/21%O₂/74%N₂) with 2 mM SIN-1 for 5, 60 or 120 min at 37°C in a modified Hanks balanced salt solution (ingredients see above) at pH 7.4.

Afterwards, cells were again washed three times with PBS buffer and an aliquot (1 ml) of the buffer from each washing step was analysed by capillary zone electrophoresis (see figure 3). L-929 cells were then lysed with 150 µl of 8% metaphosphoric acid and the lysate was centrifugated at 7500 x g for 5 min at room temperature.

The amounts of SIN-1 and SIN-1C were determined within the supernatant fraction (400 µl) of each: a) the incubation buffer, b) the PBS buffer samples after the washing steps and c) the cell lysate. The separation conditions for capillary zone electrophoresis were as follows: fused silica capillary (50 cm effective length, 75 µm internal diameter), hydrodynamic injection for 5 s, temperature 23°C, voltage 20 kV, normal polarity, UV detection at 214 nm, and 100 mM $\text{NaHPO}_4/\text{Na}_2\text{PO}_4$,

(pH 6.3) as electrolyte system; μ AMP: ca. 85, outlet: 254 nm (Lomonosova, E., et al. 1998). Concentrations of SIN-1 and SIN-1C were calculated from respective standards prepared either with PBS or 8% metaphosphoric acid. Control experiments with L-929 cells in the absence of SIN-1 were performed under the same conditions.

Migration times in capillary zone electrophoresis for SIN-1 (SIN-1C) were 4.6-4.9 min (7.3-7.7 min) in PBS and 5.8-6.3 min (11.8-13.2 min) in 8% metaphosphoric acid, respectively.

3.1.1 Determination of cellular protein content

The protein content of the biological samples was determined using an assay introduced by Bradford (Bradford, M. M. 1976) with bovine serum albumine as standard. The reagent solution is 100 mg Coomassie brilliant blue G-250 solution in 50 ml 96% ethanol, which is mixed with 100 ml 85% phosphor acid and 850 ml water. Afterwards, the mixture was purified by filtration. 3 ml of this Bradford reagent was added to 100 μ l probe (10-100 μ g protein) and after 10 min the optical density was read at 595 nm. In case of 1-10 μ g protein the microanalysis is common using 1 ml Bradford reagent added to 100 μ l probe.

Bovine serum albumine was used as a standard with 1 mg/1 ml H₂O, so that 1 μ g/ μ l protein content could be calculated. First of all, a calibration curve was made, afterwards samples were measured.



Figure 3: Capillary zone electrophoresis (Beckmann P/ACE 5000; Beckman, Munich, Germany)



Figure 4: Digital fluorescence microscope (Axiovert 135 TV; Zeiss, Oberkochen, Germany)



Figure 5: Amperometric NO sensitive electrode (ISO-NO; World Precision Instruments, Sarasota, Florida, USA)

3.2 *NAD(P)H fluorescence measurement*

3.2.1 Recording of cellular NAD(P)H fluorescence

Cellular NAD(P)H fluorescence was recorded with a digital fluorescence microscope (Axiovert 135 TV; Zeiss, Oberkochen, Germany) see figure 4 equipped with the Attofluor imaging system (Atto Instruments, Rockville, MD, USA) as described previously (Petrat, F., et al. 2003, Petrat, F., et al. 2003). Measurements were performed at 37°C using an excitation filter of $\lambda_{\text{exc.}} = 365 \pm 12.5$ nm, and monitoring the emission at $\lambda_{\text{em.}} = 450\text{-}490$ nm with a bandpass filter. Cellular NAD(P)H fluorescence was recorded at 120 s intervals with an excitation period of 0.3 s and the intensity of the exciting mercury short arc photo optic lamp attenuated 40% using gray filters to minimize photochemical effects. Single cell fluorescence was determined by manually confining the regions of interest to individual cells.

After establishing NAD(P)H baseline fluorescence (6-10 min in buffer A), SIN-1, SIN-1C, or spermine NONOate (2 mM each) were added to the cells and the temperature was held at 37°C during the experiment.

3.2.2 Determination of the subcellular distribution of NAD(P)H

A laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany) equipped with an UV laser was used (Petrat, F., et al. 2003). The glass coverslips with adherent cells were transferred to a modified Pentz chamber and cells were washed three times with PBS (37°C). Afterwards, the cells were covered with the buffer A and the experiments were performed at room temperature. Subcellular NAD(P)H distribution was determined from its subcellular fluorescence at $\lambda_{\text{exc.}} =$

365 nm and $\lambda_{em.} = 385-470$ nm. The objective lens was a 63 x NA 1.40 Plan-Apochromat. The scanning parameters were as follows: The pinhole was set at 130 μ m, producing confocal optical slices of less than 1.0 μ m thickness. Confocal images (scanning time: 7.8 s-2.5 min; zoom-factor: 0.7 to 2.5) were collected at different intervals and with different parameters. The power of the laser was set at 1-20% of intensity in order to minimize photochemical damage of the cells and bleaching/redistribution of NAD(P)H fluorescence. Some cases of very dark images required electronic brightening of the images. Image processing and evaluation were performed using the “physiology evaluation” software of the LSM 510 imaging system (glow scale procedure).

3.3 Recording of the intracellular generation of reactive nitrogen-oxide species using DAF

The intracellular production of reactive nitrogen-oxide species other than NO (e.g. N_2O_3) was visualized with the scavenger diaminofluorescein DAF-2.

The substance DAF-2DA is able to pass cell membranes. Intracellular esterases convert DAF-2DA into DAF-2 that is not able to pass cell membranes. In the presence of reactive nitrogen-oxide species DAF-2 is converted into the fluorescence dye DAF-2T. This substance is also membrane impermeable.

DAF-2 and DAF-2T have an almost identical absorbance and emission maximum as well but the quantum yield of fluorescence intensity of the DAF-2T fluorescence is more than 180 fold higher than that of DAF-2. Thus the autofluorescence of DAF-2 is negligible in regard to the high fluorescence of DAF-2T (Espey, M. G., et al. 2001, Räthel, T. R. and Dirsch, V. M. 2003).

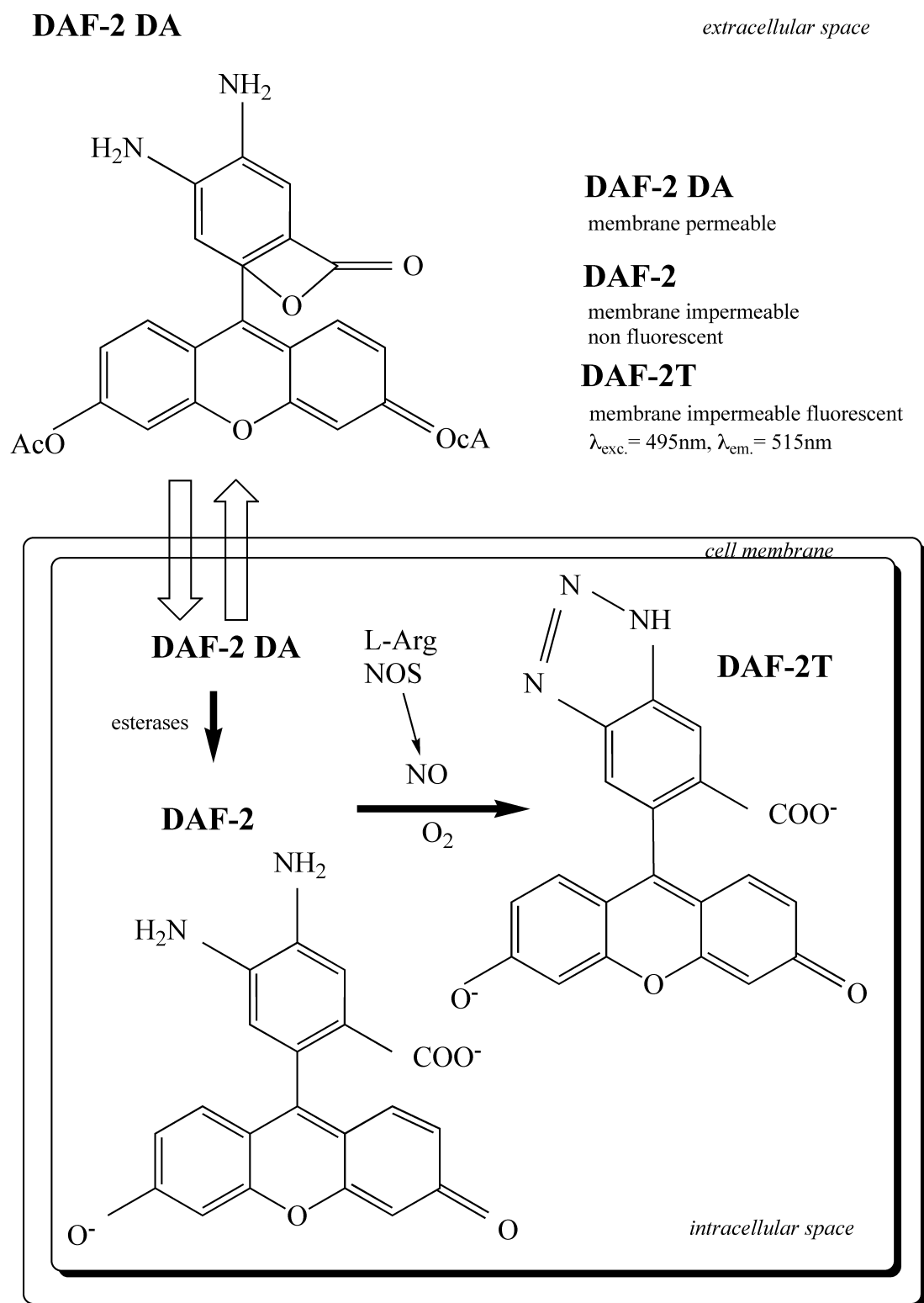


Figure 6: The fluorescence dye DAF-2: The scavenger diaminofluorescein DAF-2DA is able to pass cell membranes. Intracellular esterases convert DAF-2DA into DAF-2 that is not able to pass

cell membranes. In presence of reactive nitrogen-oxide species DAF-2 is converted into the fluorescence dye DAF-2T. This substance is also membrane impermeable.

Glass coverslips with adherent cells were transferred to a modified Pentz chamber and cells were washed three times with warm (37°C) PBS. Cells were kept with 2 μ M DAF-2DA for 1 h in MEM in the dark, as it is sensitive to light. Afterwards the cells were washed three times with PBS and covered with buffer A (see above). Measurements were performed at 37°C using a digital fluorescence microscope (see figure 4) with $\lambda_{\text{exc.}} = 488 \pm 5$ nm, and monitoring the emission at $\lambda_{\text{em.}} = 520 \pm 20$ nm using a band-pass filter. When laser microscopy was used, DAF-2 fluorescence was excited at $\lambda_{\text{exc.}} = 488$ nm and collected through a $\lambda_{\text{em.}} = 505$ nm long pass filter. After establishing the intracellular DAF-2 baseline fluorescence (6-10 min), SIN-1 (2 mM) was added from a concentrated stock solution (see above).

3.4 Determination of nitric oxide with a NO sensitive electrode

Extracellular nitric oxide formation was determined using a polarographic amperometric NO sensitive electrode (ISO-NO; World Precision Instruments, Sarasota, Florida, USA) see figure 5 as described in Ref. (Tsukahara, H., et al. 1993). During the measurements the temperature was strictly regulated at $37 \pm 1^\circ\text{C}$ and solutions were continuously stirred during the experiments. The electrode was calibrated and the generated NO was quantified according to the instructions of the manufacturer using potassium iodide (100 mM) in H_2SO_4 (0.1 M) as a calibration solution to which various amounts of NaNO_2 (50 μ M) were added.

The calibration procedure was daily repeated, and plastic vessels were avoided. Measurements were performed either in a cell-free system or with L-929 mouse

fibroblasts in modified Pentz chambers with buffer A (pH = 7.4) in a 100% humidified atmosphere of 5%CO₂/21%O₂/74%N₂. After establishing the baseline level of the NO electrode SIN-1 or SIN-1C (2 mM each) were added and the NO concentrations recorded.

3.5 Intracellular NO measurement with the Fluorescent Nitric Oxide Cheletropic Trap

Intracellular generation of NO in L-929 mouse fibroblasts was verified with the Fluorescent Nitric Oxide Cheletropic Trap FNOCT-1, which directly and specifically traps intracellular NO (Meineke, P., et al. 1999, Meineke, P., et al. 2000). This method allows a sensitive and quantitative detection of nitric oxide production in biological samples, as it does not cause cell damage. Scavenging of NO converts FNOCT-1 into the stable, fluorescent adduct NOCT-NOH.

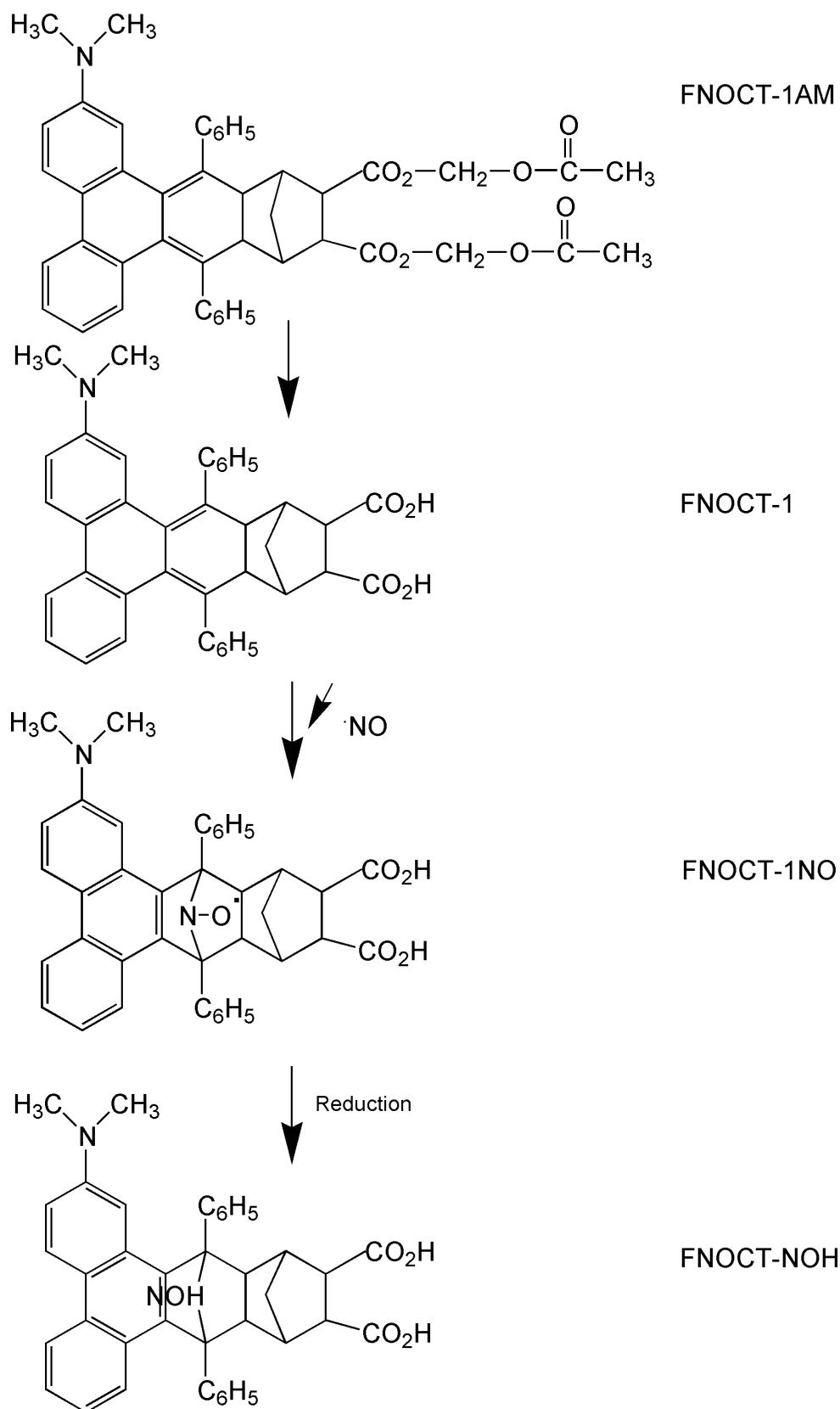


Figure 7: Scavenging of NO converts the fluorescent nitric oxide cheletropic trap FNOCT-1 into the stable, fluorescent adduct, FNOCT-NOH.

Confluent L-929 mouse fibroblasts were washed three times with PBS (37°C) and incubated for 1 h with FNOCT-1AM (50 μ M) in buffer A, that additionally contained pluronic (0.1%), probenecid (1 mM), glucose (5 mM) and DMSO (0.5%). The membrane-permeating acetoxymethylester derivate FNOCT-1AM is intracellularly hydrolyzed to the membrane-impermeable compound FNOCT-1 by intracellular hydrolases.

Afterwards intracellular FNOCT-1 fluorescence was recorded using a digital fluorescence microscope (see figure 4) at $\lambda_{\text{exc.}} = 380 \pm 10$ nm and $\lambda_{\text{em.}} = 450\text{-}490$ nm at 120 s intervals with an excitation period of 0.3 s. Single-cell fluorescence was determined by confining the regions of interest manually to individual cells and the temperature was held at 37°C during the experiments.

After establishing the baseline fluorescence (6-10 min), SIN-1 or SIN-1C (2 mM each) was added.

3.6 Cell viability

The uptake of the dye propidium iodide (5 μ g/ml) was routinely determined either during or at the end of the experimental procedures in order to detect loss of cell viability. The red fluorescence of propidium iodide excited at 543 nm was collected through a 560 nm long-pass filter when laser scanning microscopy was used; using digital fluorescence microscopy, propidium iodide was detected at $\lambda_{\text{exc.}} = 535 \pm 17.5$ nm and $\lambda_{\text{em.}} \geq 590$ nm.

The exclusion of propidium iodide and the fact that intracellular substances like DAF-2T and NAD(P)H did not leak out of the cells during the duration of the experiments indicated that the plasma membrane remained intact.

3.7 *Statistics*

All experiments were repeated at least three times. Cellular microfluorographs and traces shown in the figures are representative of experiments performed. The results are expressed as means \pm S.D.

4 RESULTS

4.1 Evidence that SIN-1 penetrates L-929 cells

In order to demonstrate that SIN-1 is capable of passing through membranes, 2 mM of the sydnonimine was added to L-929 cells and experiments performed as described above. The amounts of SIN-1 and SIN-1C were determined within the supernatant fraction (400 μ l) of each: a) the incubation buffer, b) the PBS buffer samples after the washing steps and c) the cell lysate. One representative capillary zone electrophoresis chromatogram of the last washing solution and one representative chromatogram of a cell lysate are shown in figures 8A and 8B. From each chromatogram the intracellular content of SIN-1 per mg protein was determined. The result is shown in figure 8C.

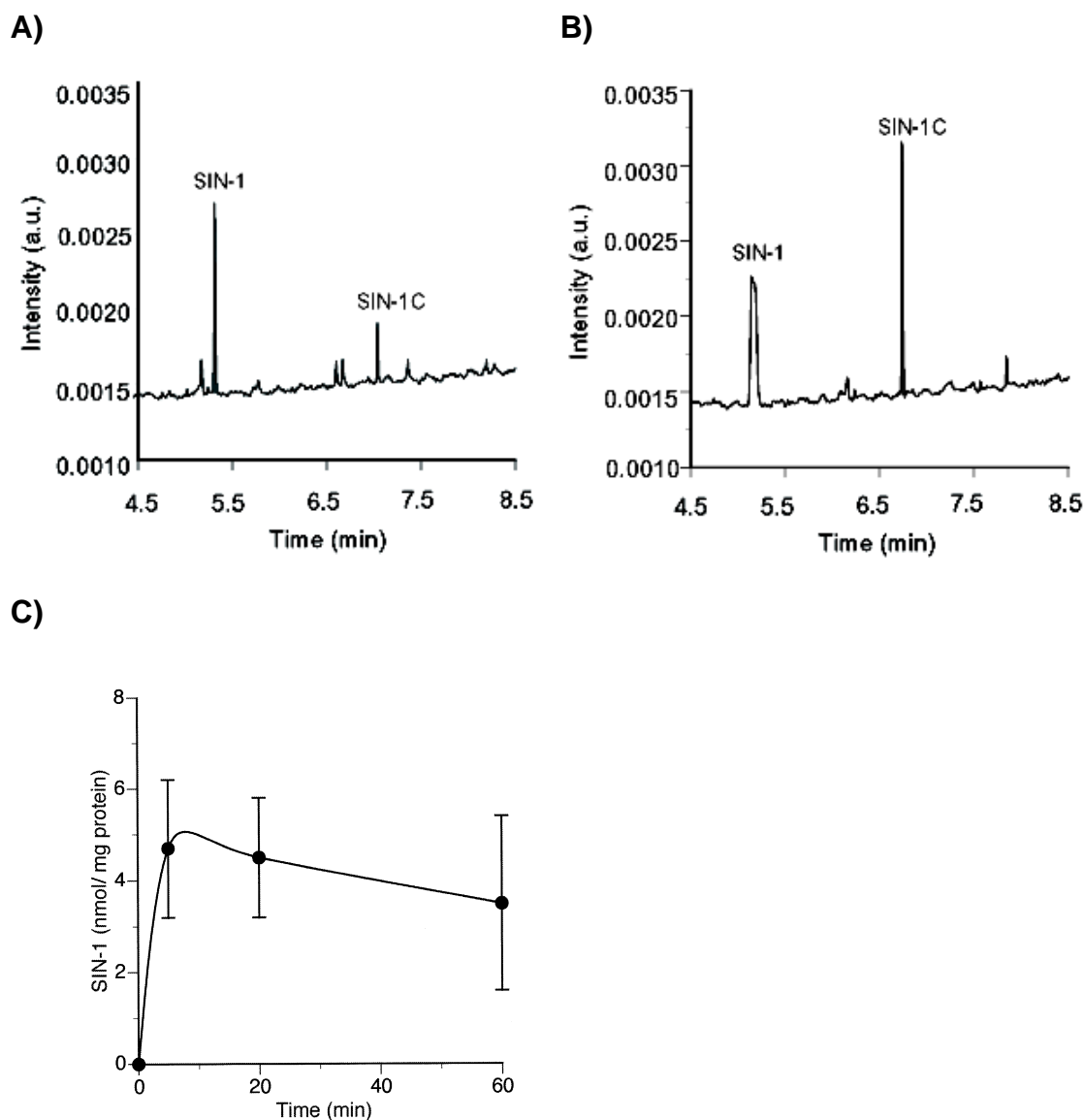


Figure 8: L-929 mouse fibroblasts were cultured in MEM on 176 cm² plastic flasks 48 h before starting the experiments. On the day of the experiment, the cells were washed with PBS and incubated with SIN-1 (2 mM) in a modified Hanks balanced salt solution at pH 7.4 (37°C). After repeating washing the L-929 cells were lysed with 150 μ l of 8% metaphosphoric acid. An aliquot of the cell lysate was taken and the amount of SIN-1 and SIN-1C analyzed by capillary zone electrophoresis. SIN-1 and SIN-1C were identified by spiking with authentic material.

A) A typical chromatogram of a last washing solution is shown.

B) Figure 8B shows a typical chromatogram of the L-929 cell lysate.

C) Figure 8C shows the intracellular amount of SIN-1 per mg protein after an incubation of 5, 20 and 60 min. Values shown in Figure 8C represent means \pm S.D. of three experiments using L-929 mouse fibroblasts.

Already after 5 min of incubation about 5 nmol SIN-1 per mg protein was detected in L-929 cells. At prolonged incubation periods, the intracellular content of SIN-1 decreased moderately. This decrease did not correspond with the known half-life of SIN-1, $t_{1/2}(\text{SIN-1}) = 40 \text{ min}$, in purified buffer solutions (Lomonosova, E., et al. 1998, Schrammel, A., et al. 1998). However, such a decreased decay has been already reported by Meinertz *et al.* (Meinertz, T., et al. 1985). From a SIN-1 content of about 5 nmol/mg protein the concentration was estimated with the assumption that the volume of a single fibroblast is 3.5 pL (Dall'Asta, V., et al. 1994, Dall'Asta, V., et al. 1994, Mastrocola, T., et al. 1993) yielding an intracellular concentration of SIN-1 of $0.9 \pm 0.3 \text{ mM}$, *i.e.* approximately half the concentration in the supernatant (2 mM) respectively.

Intracellular SIN-1C could only be found at marginal contents (about 55, 110, and 85 pmol SIN-1C per mg protein after 5, 60, and 120 min).

Hence, these data clearly demonstrated that SIN-1 but not SIN-1C rapidly penetrates L-929 cells.

4.2 Evidence for an intracellular production of reactive nitrogen-oxide species from SIN-1 in L929 cells

Since diffusion of SIN-1 into L-929 cells is quite rapid, we tried to scavenge possible intracellularly produced reactive nitrogen-oxide species with the scavenger DAF-2DA (2 μM) as a fluorescent indicator. DAF-2 reacts with such reactive intermediates to yield the fluorescent dye DAF-2T (Espey, M. G., et al. 2001). After addition of 2 mM SIN-1 to L-929 cells, a strong, continuous increase in the fluorescence of DAF-2T could be monitored by digital fluorescence microscopy

($\lambda_{\text{exc.}} = 488 \pm 10$ nm; $\lambda_{\text{em.}} = 520 \pm 20$ nm) during the first 15 min. Figure 9 shows the curve of this registration.

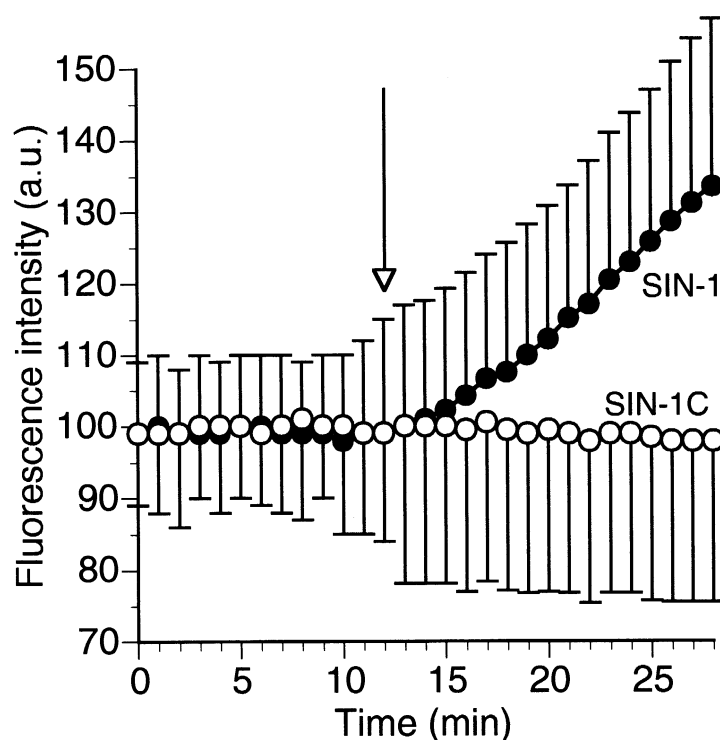


Figure 9: Detection of intracellular reactive nitrogen-oxide species generated by SIN-1 using digital fluorescence microscopy

The generation of reactive nitrogen-oxide species in cultured L-929 cells was monitored by digital fluorescence microscopy ($\lambda_{\text{exc.}} = 488 \pm 10$ nm; $\lambda_{\text{em.}} = 520 \pm 20$ nm) utilizing 4,5-diaminofluorescein diacetate DAF-2 (2 μM) as a fluorescence indicator. Upon reaction of non-fluorescent DAF-2 with intracellular reactive nitrogen species the fluorescent product DAF-2T is intracellularly generated. L-929 cells were cultured on glass coverslips, washed three times with PBS and incubated for 1 h with the membrane-permeable compound DAF-2DA in MEM. Afterwards, cells were washed again three times with PBS and then covered with buffer A, pH 7.4 (37°C). After establishing the baseline fluorescence, SIN-1 or SIN-1C (2 mM each) was added. Values shown represent means \pm S.D of three experiments using L-929 mouse fibroblasts from different subcultures. The arrow indicates the addition of either SIN-1 or of SIN-1C.

In control experiments performed with the pharmacologically inactive compound SIN-1C (2 mM), DAF-2T fluorescence did not increase, as also shown in figure 9. Data taken with the laser scanning microscope clearly demonstrate an increase of fluorescence intensity (figure 10). The stable level of fluorescence intensity is shown from photo 1-4. At 30 minutes SIN-1 was added and the increase of fluorescence intensity is visible.

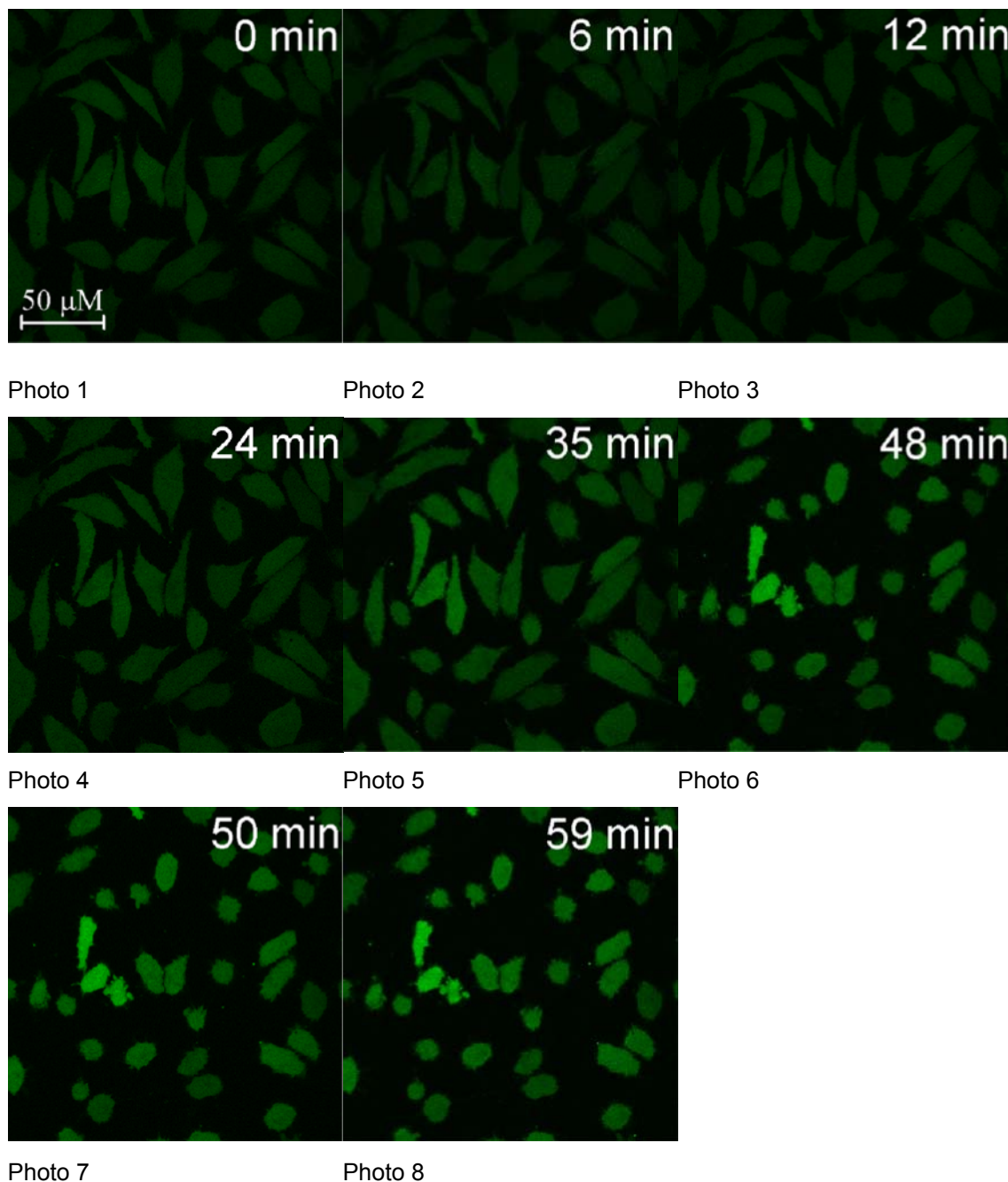


Figure 10: Detection of intracellular reactive nitrogen-oxide species generated by SIN-1 using laser scanning microscopy. The generation of reactive nitrogen-oxide species in cultured L-929 cells was visualized by laser scanning microscopy ($\lambda_{\text{exc.}} = 488 \pm 10 \text{ nm}$; $\lambda_{\text{em.}} = 520 \pm 20 \text{ nm}$) utilizing 4,5-diaminofluorescein diacetate DAF-2 ($2 \mu\text{M}$) as a fluorescence indicator. Upon reaction of non-fluorescent DAF-2 with intracellular reactive nitrogen species the fluorescent product DAF-2T is intracellularly generated. L-929 cells were cultured on glass coverslips, washed three times with PBS and incubated for 1 h with the membrane-permeable compound DAF-2DA in MEM. Afterwards, cells were washed again three times with PBS and then covered with buffer A, pH 7.4 (37°C). After establishing the baseline fluorescence shown in photo 1-4, SIN-1 (2 mM) was added at time point 30 min. The following pictures show the increase of fluorescence intensity.

These experiments indicated that RNOS like N_2O_3 or NO_2 are intracellularly produced from SIN-1 in L-929 cells.

4.3 Evidence for the generation of freely diffusing NO from intracellularly operating SIN-1 in L-929 cells

4.3.1 Nitric oxide sensitive electrode

Because the DAF-2 nitrosating intermediates should have been derived from initially released NO at pH 7.0-7.5, we monitored the production of nitric oxide from SIN-1 using a polarographic amperometric NO-sensitive electrode. NO was quantified on basis of daily performed calibrations as explained above.

4.3.1.1 Cell free system

In the absence of L-929 cells, SIN-1-dependent formation of freely diffusing nitric oxide was not detectable in regard to the detection limit of 1 nM. Figure 11 shows the comparison with nitric oxide release in cell free and cellular system. Obviously, the release of nitric oxide was undetectable in a cell free system.

Nitric oxide release after addition of 2mM SIN-1
in a cell free system and in L-929 cells.

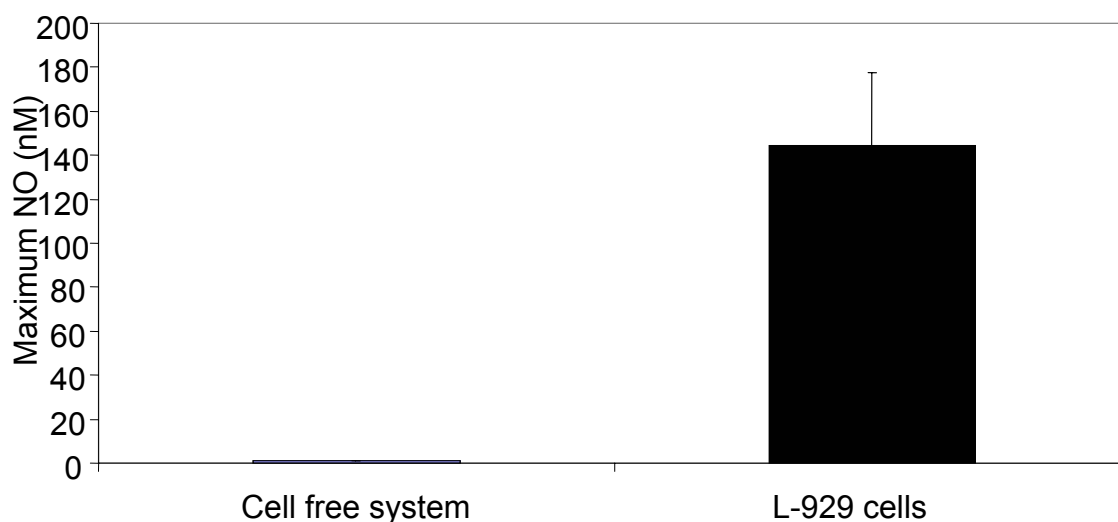


Figure 11: Electrochemically monitored formation of nitric oxides from SIN-1

Formation of nitric oxide was detected by using a polarographic amperometric NO sensitive electrode. NO was quantified on basis of daily performed calibrations. Formation of NO from SIN-1 (2 mM) was measured at 37°C within chemical - cell free system - and in the supernatant (buffer A) of L-929 cells. Glass coverslips with adherent cells were transferred to a modified Pentz chamber and cells washed three times with 2 ml warm (37°C) PBS buffer before buffer A was added. Values shown in Figure 11 represent means \pm S.D of three experiments using L-929 mouse fibroblasts from different subcultures.

4.3.1.2 Cell system

Formation of NO from SIN-1 (2 mM) was additionally measured at 37°C within the supernatant (buffer A) of L-929 cells. The development of nitric oxide release was recorded and the results are shown in Figure 12. In the presence of L-929 cells (4×10^5 cells/ cm^2), however, formation of NO could be monitored with an initial rate of 10.2 ± 5.9 nM/ min. A “plateau” concentration of ca. 160 nM NO was established after 45 min (Fig. 12). These observations are at variance with data of Haddad *et al.* (Haddad, I. Y., et al. 1996) who found that alveolar type II cells did not release

any freely diffusing nitric oxide after incubation with SIN-1. Such a discrepancy may well be due to the inherent metabolism of the cells.

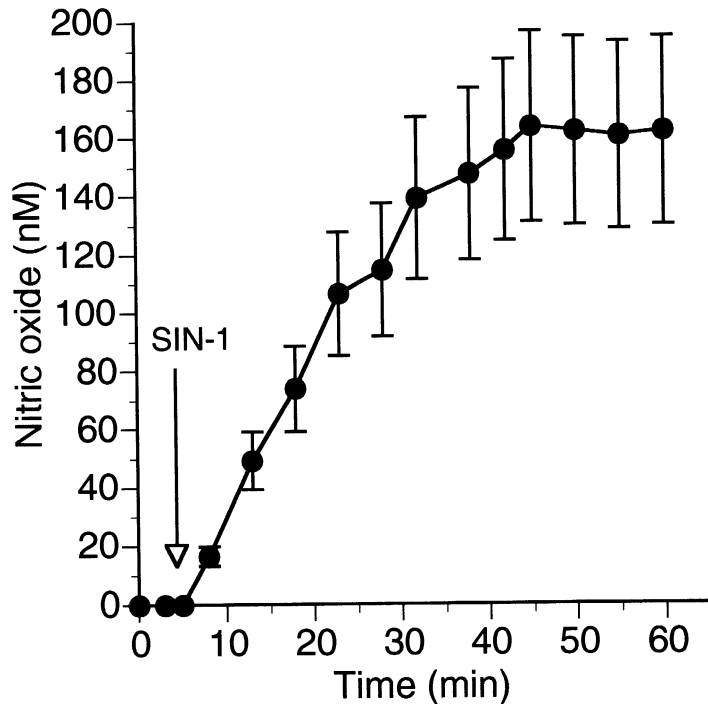


Figure 12: Electrochemically monitored formation of nitric oxides from SIN-1

Formation of nitric oxide was detected by using a polarographic amperometric NO sensitive electrode. NO was quantified on basis of daily performed calibrations. Formation of NO from SIN-1 (2 mM) was measured at 37°C within chemical - cell free-system - and in the supernatant (buffer A) of L-929 cells. Glass coverslips with adherent cells were transferred to a modified Pentz chamber and cells washed three times with 2 ml warm (37°C) PBS buffer before buffer A was added. The trace shown indicates the SIN-1 dependent formation of nitric oxide in the supernatant of the L-929 cell cultures showing an initial rate of 10.2 ± 5.9 nM/min and a plateau concentration of ca. 160 nM NO. Values shown represent means \pm S.D. of three experiments using L-929 mouse fibroblasts from different subcultures.

4.3.2 NO-scavenger F-NOCT

To underline and verify the experiments performed with the NO-electrode, formation of nitric oxide was intracellularly measured by using the NO scavenger FNOCT-1. This compound is highly specific for NO (Meineke, P., et al. 1999, Meineke, P., et al. 2000), in contrast to DAF-2, which reacts with reactive nitrogen

species but did not scavenge NO (Espey, M. G., et al. 2001). Scavenging of NO converts FNOCT-1 into the stable, fluorescent adduct, FNOCT-NOH. On addition of 2 mM SIN-1 to L-929 cells preloaded with FNOCT-1AM, the intracellular fluorescence of FNOCT-NOH could be detected, the intensity increasing linearly with time, thus confirming the trapping of freely diffusing NO as shown in Figure 13.

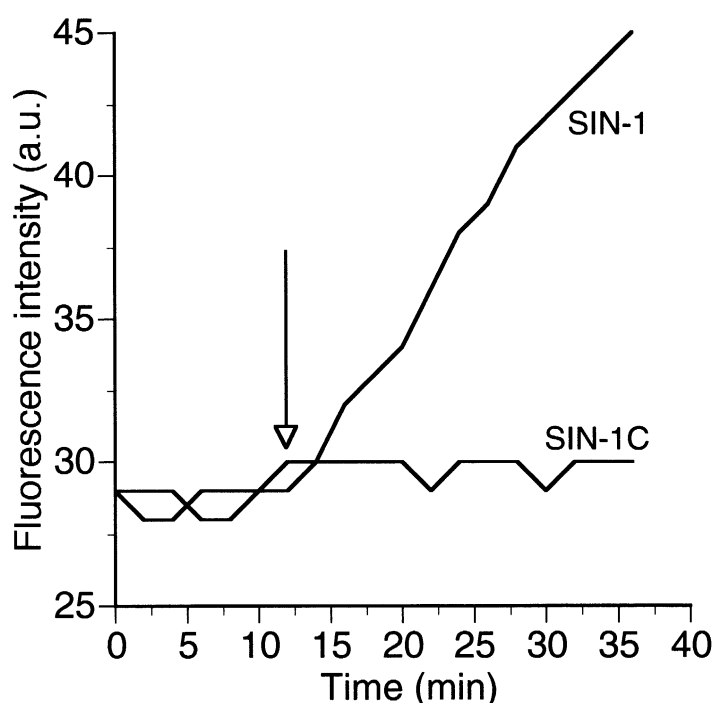


Figure 13: SIN-1-dependent formation of NO in L-929 cells as determined with the FNOCT-1 method

Cells were cultured on glass coverslips, transferred to a modified Pentz chamber, then washed three times with PBS buffer and then incubated with FNOCT-1AM (50 μ M) for 1 h in MEM (37°C). Afterwards, cells were washed again with PBS buffer and covered with buffer A (37°C). The intracellular fluorescence of FNOCT-1 ($\lambda_{\text{exc.}} = 380 \pm 10$ nm, $\lambda_{\text{em.}} = 450\text{-}490$ nm) was recorded at 120 s intervals with an excitation time of 0.3 s using digital fluorescence microscopy. After establishing the baseline fluorescence, SIN-1 or SIN-1C (2 mM each) was added (arrow). Data are representative for at least 5 experiments using L-929 mouse fibroblasts from different subcultures.

On the other hand, the background fluorescence level of FNOCT-1AM loaded cells was hardly affected by SIN-1C (2 mM). Control experiments performed with SIN-1 (0.025-1 mM) and FNOCT-1 (50 μ M) in the absence of L-929 cells validated the expectation that the reaction of NO with FNOCT-1 ($k(^{\bullet}\text{NO} + \text{FNOCT-1}) = 170 \pm 10 \text{ M}^{-1} \text{ s}^{-1}$) (Meineke, P., et al. 1999, Meineke, P., et al. 2000) cannot compete with the fast reaction of nitric oxide with $\text{O}_2^{\bullet-}$ ($k(^{\bullet}\text{NO} + \text{O}_2^{\bullet-}) = \sim 5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) (Kissner, R. and Koppenol, W. H. 2002, Ross, A. B., et al. 1998) to form peroxynitrite because of the missing FNOCT-NOH fluorescence in these experiments (data not shown). Notably, the FNOCT-1/FNOCT-NOH fluorescence intensity was found to be orders of magnitude higher than the cellular NAD(P)H fluorescence intensity (data not shown), which therefore did not contribute significantly to the changes in the observed fluorescence intensity. Thus, the L-929 cells initiated the release of nitric oxide from intracellularly decaying SIN-1.

4.4 Evidence that the intracellular NAD(P)H level is influenced by both NO and RNOS in L-929 cells

Nitric oxide is known to induce an increase in the intracellular, especially the intramitochondrial concentration of NAD(P)H by blocking the mitochondrial electron transport chain due to reaction with both the heme a_3 and copper Cu_B group of cytochrome oxidase (Cooper, C. E. 2002, Horie, Y., et al. 1997). Contrary to NO, higher reactive nitrogen-oxide species (RNOS) like NO_2 and N_2O_3 are expected to diminish the intracellular level of NAD(P)H via two pathways, i.e. indirectly due to an enhanced oxidation of glutathione (Heales, S. J. R. and Bolanos, J. P. 2002,

Radi, R., et al. 1991, Sies, H., et al. 1997, Tu, B., et al. 1995) and its regeneration by the glutathione reductase/NADPH pathway (Kirsch, M. and de Groot, H. 2001) and directly via attack by $\text{CO}_3^{\cdot-}$ and NO_2 radicals (Kirsch, M. and de Groot, H. 1999) produced from peroxynitrite in the presence of CO_2 . In order to answer the question whether intracellular SIN-1 primarily affects L-929 cells via NO induced inhibition of the respiratory chain (indicated by an increase of NAD(P)H) or via RNOS (indicated by a decrease of NAD(P)H), the influence of SIN-1 on total cellular and subcellular NAD(P)H fluorescence was recorded using digital fluorescence microscopy as well as laser scanning microscopy.

4.4.1 Total intracellular NAD(P)H amount

As observed by digital fluorescence microscopy, addition of SIN-1 (2 mM) to L-929 cells resulted in a 40% decrease of the total NAD(P)H fluorescence within 15 min, thus indicating a dominating intracellular action of the RNOS as shown in Figure 14. On the other hand, the NO donor compound Spermine NONOate (2 mM) increased the total intracellular NAD(P)H fluorescence by ~12% (Fig. 14), as expected for a sole production of NO. In control experiments, addition of KCN (1 mM), a well-known inhibitor of the respiratory chain, led to a comparable increase in the total NAD(P)H fluorescence intensity (data not shown).

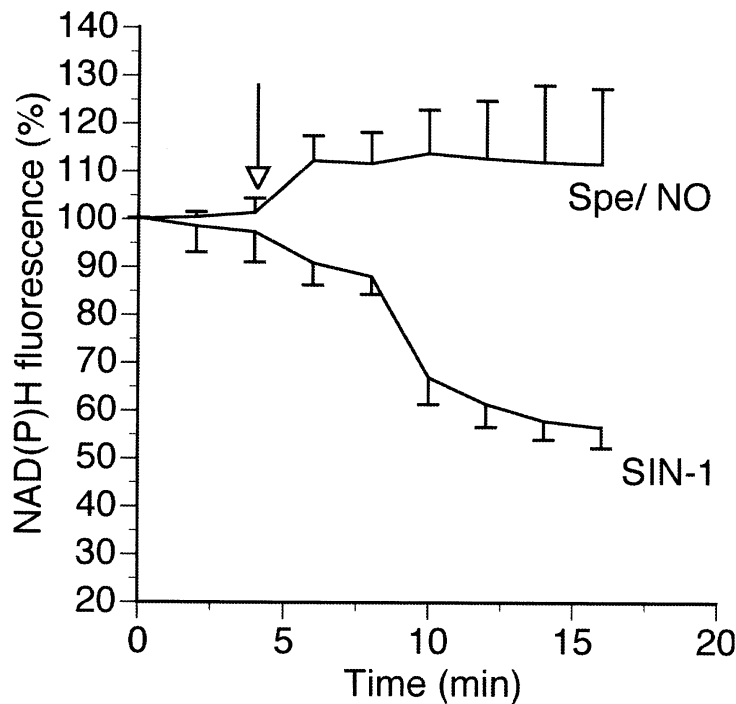


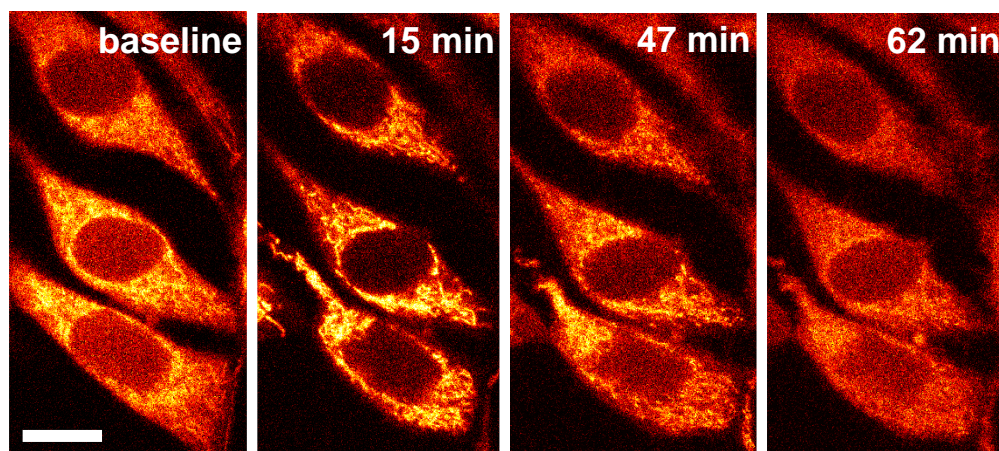
Figure 14: Effect of SIN-1 and spermine NONOate on the cellular NAD(P)H fluorescence of L-929 cells

L-929 mouse fibroblasts were cultured on glass coverslips, transferred to a modified Pentz chamber and washed three times with PBS buffer. Afterwards, cells were covered with buffer A (37°C) and fluorescence measurements were started. Cellular NAD(P)H fluorescence was recorded at 120 s intervals using digital fluorescence microscopy ($\lambda_{exc.} = 365 \pm 12.5$ nm; $\lambda_{em.} = 450-490$ nm). After establishing the baseline fluorescence (5 min), SIN-1 (2 mM) or spermine NONOate (2 mM) were added (arrow) to the buffer. The traces shown represent means \pm S.D. of 5 experiments using L-929 mouse fibroblasts from different subcultures.

4.4.2 Influence on subcellular NAD(P)H level

In order to verify the effect of SIN-1 on the total cellular NAD(P)H level and to elucidate possible changes of NAD(P)H fluorescence within the various subcellular compartments, high resolution laser scanning microscopy investigations were performed. The effect of SIN-1 (2 mM) on subcellular NAD(P)H fluorescence ($\lambda_{exc.} = 365$ nm; $\lambda_{em.} = 385-470$ nm) was imaged at the time points indicated using a laser scanning microscope equipped with an UV laser.

A)



B)

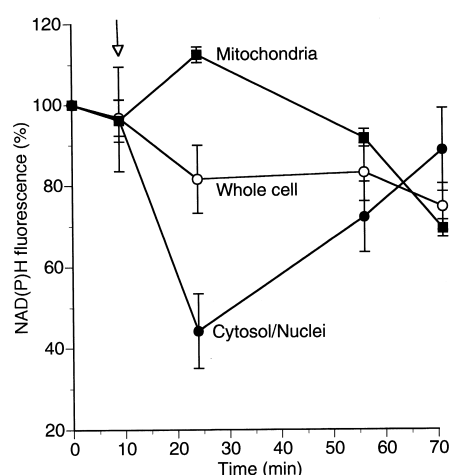


Figure 15: Effect of SIN-1 on subcellular NAD(P)H fluorescence of L-929 cells

L-929 mouse fibroblasts were cultured on glass coverslips, transferred to a modified Pentz chamber and washed three times with PBS buffer. Afterwards, L-929 mouse fibroblasts were covered with buffer A (37°C) and fluorescence measurements were started. The effect of SIN-1 (2 mM) on subcellular NAD(P)H fluorescence ($\lambda_{\text{exc.}} = 365 \text{ nm}$; $\lambda_{\text{em.}} = 385\text{-}470 \text{ nm}$) was imaged at the time points indicated using a laser scanning microscope equipped with an UV laser.

A) shows images of the whole cells in glow scale. Image processing and evaluation performed with the “physiology evaluation” software of the LSM 510 imaging system are called glow scale procedure. Bar shown in (A) indicates 10 μm

B) shows the time evolution of the NAD(P)H fluorescence intensities of the mitochondria, nuclei and whole cells as shown in the microfluorographs above. Data shown represent means \pm S.D. of 3 experiments using L-929 mouse fibroblasts from different subcultures.

In Figure 15A changes upon addition of SIN-1 are shown in the subcellular distribution of NAD(P)H in L-929 cells, respectively. Traces shown in Figure 15B represent the corresponding fluorescence intensity of NAD(P)H as evaluated within the mitochondria, nuclei, and cytosol of L-929 fibroblasts.

In these experiments, addition of 2 mM SIN-1 to L-929 cells led to a small decrease (about 20% within 15 min) of the total cellular NAD(P)H fluorescence (Fig 15A,B). This apparent disagreement to the experiments performed with the digital microscopy (Fig. 14) might be explained by the SIN-1-induced morphological alterations of the L-929 cells. These alterations may significantly affect the detection of cellular NAD(P)H fluorescence when digital fluorescence microscopy is used but not when laser scanning microscopy is applied because of optical sectioning with this confocal system. In any case, a SIN-1-induced decrease of cellular NAD(P)H was obtained with both digital microscopy and laser scanning microscopy.

Evaluation at subcellular levels revealed that SIN-1 decreased the initial cytosolic and nuclear NAD(P)H fluorescence intensity by about 60% within the first 15 min. On the other hand, mitochondrial NAD(P)H fluorescence was increased by ~ 12% (Figs. 15A and 15B), in excellent agreement with the total increase of cellular NAD(P)H obtained with the NO donor compound spermine/NO (Fig. 14). These observations strongly indicate that SIN-1 diametrically influenced the intracellular NAD(P)H concentrations of L-929 cells by both RNOS and nitric oxide. During prolonged incubation (ca. 70 min) mitochondrial NAD(P)H fluorescence continuously decreased to reach about 70% of its initial level, whereas the NAD(P)H fluorescence in the cytosol and nuclei was fully restituted (Fig. 15B).

5 DISCUSSION

There is only very little doubt that in the absence of efficient traps for nitric oxide and superoxide SIN-1 almost quantitatively generates peroxynitrite via diffusion-controlled radical recombination of NO and $O_2^{\cdot-}$ in cell-free systems (Kelm, M., et al. 1997, Kirsch, M., et al. 1998). In accord, only very low levels of NO (and NO_2) could be detected by means of cheletropic NO Traps (NOCTs) (Korth, H.-G., et al. 1994). Because of this, SIN-1 is often used in toxicity studies for the treatment of cells with extracellularly generated peroxynitrite (Bouton, C., et al. 1997, Chun-Qi, L. 2002, Doulias, P. T., et al. 2001, Garcia-Nogales, P., et al. 2003, Gergel, D., et al. 1995, Kaji, T., et al. 2002). In fact, in the presence of alveolar type II cells the extracellular SIN-1-dependent formation of peroxynitrite, as demonstrated by oxidation of extracellular dihydrorhodamine123, was found to be independent of the presence of these cells (Haddad, I. Y., et al. 1996).

The experiments described here clearly indicate that SIN-1 can readily pass through cell membranes. In L-929 cells the uptake of SIN-1 was directly measured and the intracellular production of RNOS was corroborated by DAF-2 nitrosation and NAD(P)H oxidation, respectively. The exclusion of propidium iodide and the fact that neither DAF-2T nor NAD(P)H did leak out of the cells during the duration of the experiments indicated that the plasma membrane remained intact. The finding that SIN-1 may readily pass the plasma membrane of cells is in line with observations of Meinertz *et al.* (Meinertz, T., et al. 1985), who detected SIN-1 in human plasma after molsidomine administration. Since SIN-1 is intracellularly formed from the prodrug molsidomine in the liver, SIN-1 obviously penetrates the

plasma membrane of hepatocytes with an efficiency similar to the one observed here for L-929 cells.

The intracellular formation of freely diffusing NO from SIN-1 ($[\text{NO}]_{\text{SIN-1}}$) should decisively depend on the local superoxide dismutase activity due to competition between NO and SOD for $\text{O}_2^{\cdot-}$. Because natural SOD activities strongly vary with the type of the cells (ca. 4-400 units/ml=0,025-2,5 μM) (Minami, M. and Yoshikawa, H. 1979, Oyanagui, Y. 1984), the nature and yields of the reactive nitrosating intermediates produced from intracellularly decaying SIN-1 likewise should be strongly dependent on cell type. The chemical situation in the intracellular milieu will be further complicated for cells actively producing NO via the NOS arginine pathway ($[\text{NO}]_{\text{NOS}}$). In such a case, an increased amount of $\text{O}_2^{\cdot-}$ will be trapped by endogenously produced NO rather than by SOD. Hence, SOD will be less effective in inhibiting the formation of peroxynitrite from intracellular decaying SIN-1. From the reported rate constants of $\text{O}_2^{\cdot-}$ with both NO and SOD (Kissner, R. and Koppenol, W. H. 2002, Ross, A. B., et al. 1998), one can estimate that the intracellular formation of peroxynitrite from SIN-1 is preferred at concentration ratios $[\text{NO}]_{\text{total}} / [\text{SOD}] \geq 0.1-0.5$ with $[\text{NO}]_{\text{total}} = [\text{NO}]_{\text{NOS}} + [\text{NO}]_{\text{SIN-1}}$.

The situation may be changed when the cell type (for instance bovine aortic endothelial cells (BAECs)) is highly effective in releasing NO under basal conditions, because intracellularly decaying SIN-1 is then expected to be (more or less) quantitatively converted into peroxynitrite (Christoph, S., et al. 2004). However, the decay of SIN-1 is strongly hampered in the intracellular milieu of BAECs so that the SIN-1-induced oxidation of cellular NAD(P)H could only be observed during the first initial 10 min of incubation (Christoph, S., et al. 2004).

The fact that the intracellular concentration of SIN-1 is four-fold higher in BAECs (Christoph, S., et al. 2004) than in L-929 cells may conclusively explain this decreased decay because the intracellular oxygen concentration necessary for supporting the decay of SIN-1 should be four-times faster consumed in BAECs than in L-929 cells.

As a consequence of both the endogenous nitric oxide and the hampered decay of SIN-1 in BAECs, no exogenous NO is released from SIN-1. Since intracellular SIN-1 may generate both nitric oxide and RNOS, intracellular NAD(P)H may be diametrically affected. Nitric oxide is known to inhibit the respiratory chain and to increase the cellular NAD(P)H level (Horie, Y., et al. 1997, Radi, R., et al. 2002) (Fig. 14), whereas peroxynitrite and other RNOS should be highly effective in oxidizing NAD(P)H either directly (Goldstein, S. and Czapski, G. 2000, Kirsch, M. and de Groot, H. 1999, Kirsch, M. and de Groot, H. 2001, Kirsch, M. and de Groot, H. 2000, Kirsch, M. and de Groot, H. 2002, Kuhn, D. M. and Geddes, T. J. 2002, Schmidt, H. H. H. W., et al. 1996) or indirectly by an enhanced oxidation of glutathione (Buckley, B. J. and Whorton, A. R. 2000, Everett, S. A., et al. 1996, Heales, S. J. R. and Bolanos, J. P. 2002, Kirsch, M. and de Groot, H. 2001, Kirsch, M., et al. 2002, Mayer, B., et al. 1998, Radi, R., et al. 1991, Tu, B., et al. 1995) thus resulting in a decrease of the NADPH level due to the enzymatic action of glutathione reductase (Kirsch, M. and de Groot, H. 2001). In addition, some authors propose a capability of peroxynitrite to irreversibly inhibit the respiratory chain (see for example (Radi, R., et al. 1994) and references herein). However, as in cellular systems peroxynitrite mediates its harmful effects most likely via the radicals NO_2^\cdot and $\text{CO}_3^{\cdot-}$ (Bonini, M. G., et al. 1999, Lahnig, M. 1999), and because NAD(P)H readily reacts with these radicals (Goldstein, S. and Czapski, G. 2000,

Kirsch, M. and de Groot, H. 1999, Kirsch, M. and de Groot, H. 2001), a peroxynitrite-mediated irreversible inhibition of the respiratory chain must also be accompanied by an initial decrease of mitochondrial NAD(P)H. We can safely exclude this possibility for L-929 cells, because SIN-1 increased the mitochondrial level of NAD(P)H by approximately ~12% (Fig. 15B). This possibility depends on the cell type (Christoph, S., et al. 2004), as SIN-1 initially decreased the mitochondrial NAD(P)H level in BAECs by about 20% as expected from action of peroxynitrite. Thome *et al.* (Thome, U., et al. 2003) claimed that peroxynitrite can directly attack SIN-1, thereby generating poorly characterized “bioactive” compounds. However, the experiments of Thome *et al.* are likely to be flawed by the presence of the buffer HEPES, which by attack of peroxynitrite or radicals derived from it will cause random formation of $O_2^{\cdot-}$, H_2O_2 , and a yet uncharacterized “nitric oxide donating compound” (Kirsch, M., et al. 1998, Lomonosova, E., et al. 1998, Schmidt, K., et al. 1998). Finally, an effective reaction between peroxynitrite and SIN-1 appears highly unlikely because SIN-1 (50-1000 μM) is unable to inhibit the peroxynitrite (250 μM)-mediated oxidation of NADH (200 μM) at pH 6.5 (unpublished results).

Recently, Garcia-Nogales *et al.* (Garcia-Nogales, P., et al. 2003) studied the effects of SIN-1 as a putative peroxynitrite generator on both astrocytes and neurons and observed that peroxynitrite increases the total NAD(P)H concentration in these cells by about 80%. This increase in the total cellular NAD(P)H concentration was taken as an argument to question the relevance of the observation that peroxynitrite is highly effective in oxidizing NAD(P)H *in vitro* (Kirsch, M. and de Groot, H. 1999, Kirsch, M. and de Groot, H. 2001, Kirsch, M. and de Groot, H. 2000). However, singlet oxygen can directly oxidize mitochondrial

NAD(P)H (Petrat, F., et al. 2003) and the questions arises why the even more powerful $\text{CO}_3^{\cdot-}$ radical ($k(\text{NADH} + \text{CO}_3^{\cdot-}) = 1.4 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, (Goldstein, S. and Czapski, G. 2000) should not act in a similar manner. Experiments performed with BAECs (Christoph, S., et al. 2004) suggest that an increase in NAD(P)H is obviously a secondary metabolic response of these cells to the rapid NAD(P)H consumption induced by *in situ* generated peroxynitrite from SIN-1. Remarkably, such an enhanced NAD(P)H production seems to be cell-type specific because upon addition of SIN-1 to L-929 cells the basal NAD(P)H level was increased in mitochondria but decreased in the cytosol and in the nuclei (Fig. 15B). The complexity of the influence of SIN-1 on cellular NAD(P)H homeostasis is further underlined by the observation that in L-929 the cytosolic level of NAD(P)H increases at prolonged incubation periods, apparently at the expense of mitochondrial NAD(P)H.

6 CONCLUSIONS

SIN-1 is highly effective in penetrating plasma membranes of cells but the uptake of SIN-1 and the decay rate of it in the intracellular milieu depends on the cell type. The formation of peroxynitrite from intracellularly decaying SIN-1 should be suppressed by SOD and favored by endogenously produced nitric oxide. Hence, the use of SIN-1 for cell culture studies *a priori* will affect the cells in an unpredictable manner both by extracellular generation of peroxynitrite as well as by modulation the relative levels of intracellularly produced nitric oxide and RNOS, e.g. peroxynitrite. The cellular NAD(P)H level might be used to indicate whether nitric oxide or RNOS are predominantly affecting the physiological processes in the various cell compartments under a certain experimental condition. It is therefore highly problematic to employ SIN-1 as a reliable source for peroxynitrite in cell culture experiments.

7 SUMMARY

The drug molsidomine is metabolized by hepatic esterases to SIN-1 that further reacts with molecular oxygen to yield nitric oxide and superoxide. Both radicals can recombine to give the harmful entity peroxynitrite. Consequently, SIN-1 is frequently used in cell culture studies as (an extracellularly operating) peroxynitrite generator. However, little is known about the nature of the reactive species produced intracellularly from SIN-1. In the present study I demonstrated that SIN-1 can easily penetrate cell membranes as exemplified with L-929 mouse fibroblasts. Intracellularly decaying SIN-1 partly generates nitric oxide as monitored by two independent methods, *i.e.* the fluorescent nitric oxide scavenger FNOCT-1 and the nitric oxide electrode. Moreover, reactive nitrogen-oxide species (e.g. peroxynitrite, nitrogen dioxide, dinitrogen trioxide) were intracellularly formed as detected with the scavenger DAF-2. Laser scanning microscopy revealed that in L-929 cells SIN-1-derived species initially oxidized the major fraction of the NAD(P)H within the cytosol and the nuclei, whereas the mitochondrial level of NAD(P)H was somewhat increased. This opposite behavior is conclusively explained by the capabilities of SIN-1 to simultaneously generate reactive nitrogen-oxide species (like peroxynitrite and dinitrogen trioxide) and freely diffusing nitric oxide in the intracellular milieu. These observations may lead to the conclusion that SIN-1 penetrated *in vivo* its target cells in order to stimulate the release of freely diffusing NO. Nitric oxide as well as RNOS decisively affect cellular metabolism, a fact that should be examined in cell culture systems by detecting the NAD(P)H level. In any case care should be taken in experimental systems to apply SIN-1 as an exclusively peroxynitrite-generating compound.

8 REFERENCES

1. Ahlner, J., Andersson, R. G. G., Torfgard, K. E., and Axelsson, K. L. (1991): Organic nitrate esters: clinical use and mechanisms of actions. *Pharmacol. Rev.* 43, 351-423
2. Ashai, Y., Shinozaki, K., and Nagoaka, M. (1971): Chemical and kinetic study on stabilities of 3-morpholinosydnonimine and its N-ethoxycarbonyl derivate. *Chemical and Pharmaceutical Bulletin* 19, 1079-1088
3. Bonini, M. G., Radi, R., Ferrer-Sueta, G., Ferreira, A. M. D. C., and Augusto, O. (1999): Direct EPR detection of the carbonate radical anion produced from peroxynitrite and carbon dioxide. *J. Biol. Chem.* 274, 10802-10806
4. Bouton, C., Hirling, H., and Drapier, J. C. (1997): Redox modulation of iron regulatory proteins by peroxynitrite. *J. Biol. Chem.* 272, 19969 - 19975
5. Bradford, M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254
6. Brown, G. C. (1999): Nitric oxide and mitochondrial respiration. *Biochim Biophys Acta* 1411, 351-369
7. Buckley, B. J., and Whorton, A. R. (2000): Adaptive responses to peroxynitrite: increases glutathione levels and cystine uptake in vascular cells. *Am. J. Physiol.* 279, C1168 - C1176
8. Christoph, S., Petrat, F., and de Groot, H. (2004), unpublished results
9. Chun-Qi, L. (2002): Genotoxicity, mitochondrial damage, and apoptosis in human lymphoblastoid cells exposed to peroxynitrite generated from SIN-1. *Chem. Res. Toxicol.* 15, 527 - 535
10. Cooper, C. E. (2002): Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? *Trends Biochem. Sci.* 27, 33 - 39
11. Dall'Asta, V., Rossi, P. A., Bussolati, O., and Gazzola, G. C. (1994): Response of human fibroblasts to hypertonic stress. Cell shrinkage is counteracted by an enhanced active transport of neutral amino acids. *J. Biol. Chem.* 269, 10485 - 10491
12. Dall'Asta, V., Rossi, P. A., Bussolati, O., and Gazzola, G. C. (1994): Regulatory volume decrease of cultured human fibroblasts involves

- changes in intracellular amino acid pool. *Biochim. Biophys. Acta* 1220, 139 - 145
13. Dendorfer, A. (1996): Pharmacology of nitrates and other NO donors. *Herz* 21, 38 - 49
 14. Doulias, P. T., Barbouti, A., Galaris, D., and Ischiropoulos, H. (2001): SIN-1 induces DNA damage in isolated human peripheral blood lymphocytes as assessed by single cell gel electrophoresis (comet assay). *Free Radic. Biol. Med.* 30, 679 - 685
 15. Espey, M. G., Miranda, K. M., Thomas, D. D., and Wink, D. A. (2001): Distinction between nitrosating mechanisms within human cells and aqueous solution. *J. Biol. Chem.* 276, 30085-30091
 16. Everett, S. A., Dennis, M. F., Patel, K. B., Maddix, S., Kundu, S. C., and Willson, R. L. (1996): Scavenging of nitrogen dioxide, thiyl, and sulfonyl free radicals by the nutritional antioxidant β -carotene. *J. Biol. Chem.* 271, 3988-3994
 17. Feelisch, M. (1998): The use of nitric oxide donors in pharmacological studies. *N-S Arch. Pharmacol.* 358, 113 - 122
 18. Feelisch, M., and Noack, E. (1989): Molecular aspects underlying the vasodilator action of molsidomine. *J. Cardiovasc. Pharmacol.* 14, 1-5
 19. Feelisch, M., Ostrowski, J., and Noack, E. (1989): On the mechanism of NO release from sydnonimines. *J. Cardiovasc. Pharmacol.* 14, 13 - 22
 20. Furchgott, R. F., and Zawadzki, J. V. (1980): The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288, 373 - 376
 21. Gabriel, C., Camins, A., Sureda, F. X., Aquirre, L., Escubedo, E., Pallàs, M., and Camarasa, J. (1997): Determination of nitric oxide generation in mammalian neurons using dichlorofluorescein diacetate and flow cytometry. *Pharmacol. Toxicol. Methods* 38, 93 - 98
 22. Garcia-Nogales, P., Almeida, A., and Bolanos, J. P. (2003): Peroxynitrite protects neurons against nitric oxide-mediated apoptosis: a key role for glucose-6-phosphate dehydrogenase activity in neuroprotection. *J. Biol. Chem.* 278, 864-874

23. Gergel, D., Misik, V., Ondrias, K., and Cederbaum, A. I. (1995): Increased cytotoxicity of 3-morpholinosydnonimine to HepG2 cells in the presence of superoxide dismutase: Role of hydrogen peroxide and iron. *J. Biol. Chem.* 270, 20922-20929
24. Goldstein, S., and Czapski, G. (2000): Reactivity of peroxynitrite versus simultaneous generation of NO and O_2^- toward NADH. *Chem. Res. Toxicol.* 13, 735-741
25. Granger, D. L., and Lehninger, A. L. (1982): Sites of mitochondrial electron transport in macrophage-injured neoplastic cells. *J. Cell Biol.* 95, 527-535
26. Gryglewski, R. G., Marcinkiewicz, E., Robak, J., Michalska, Z., and Madej, J. (2002): Mesoionic oxatriazoles (MOTA): NO-donating characteristics and pharmacology. *Curr. Pharm. Design* 8, 167 - 176
27. Haddad, I. Y., Zhu, S., Crow, J., Barefield, E., Gadilhe, T., and Matalon, S. (1996): Inhibition of alveolar type II cell ATP and surfactant synthesis by nitric oxide. *Am. J. Physiol.* 270, L898 - L906
28. Heales, S. J. R., and Bolanos, J. P. (2002): Impairment of brain mitochondrial function by reactive nitrogen species: the role of glutathione in dictating susceptibility. *Neurochem. Int.* 40, 469 - 474
29. Horie, Y., Wolf, R., and Granger, N. D. (1997): Role of nitric oxide in gut ischemia-reperfusion-induced hepatic microvascular dysfunction. *Am. J. Physiol.* 273, G1007 - G1013
30. Huie, R. E., and Padmaja, S. (1993): The reaction of NO with superoxide. *Free. Rad. Res. Commun.* 18, 195 - 199
31. Kaji, T., Kaieda, I., Hisatsune, T., and Kaminogawa, S. (2002): 3-Morpholinosydnonimine hydrochloride induces p53-dependent apoptosis in murine primary neural cells: A critical role for p21ras-MAPK-P19ARF pathway. *NITRIC OXIDE: Biol. Chem.* 6, 125-134
32. Kankaanranta, H., Knowles, R. G., Vuorinen, P., Kosonen, O., Holm, P., and Moilanen, E. (1997): 3-Morpholino-sydnonimine-induced suppression of human neutrophil degranulation is not mediated by cyclic GMP, nitric oxide or peroxynitrite: inhibition of the increase in intracellular free calcium concentration by N-morpholinoiminoacetonitrile, a metabolite of 3-morpholino-sydnonimine. *Mol. Pharmacol.* 51, 882 - 888

33. Karow, T. Lang-Roth., R. (2003): Allgemeine und Spezielle Pharmakologie und Toxikologie. Pulheim, Eigenverlag
34. Kelm, M., Dahmann, R., Wink, D., and Feelisch, M. (1997): The nitric oxide/superoxide assay. *J. Biol. Chem.* 272, 9922 - 9932
35. Kirsch, M., and de Groot, H. (1999): Reaction of peroxynitrite with reduced nicotinamide nucleotides, formation of hydrogen peroxide. *J. Biol. Chem.* 274, 24664-24670
36. Kirsch, M., and de Groot, H. (2001): NAD(P)H, a directly operating antioxidant? *FASEB J.* 15, 1569-1574
37. Kirsch, M., and de Groot, H. (2000): Ascorbate is a potent antioxidant against peroxynitrite-induced oxidation reactions. *J. Biol. Chem.* 275, 16702 - 16708
38. Kirsch, M., and de Groot, H. (2002): Formation of peroxynitrite from reaction of nitroxyl anion with molecular oxygen. *Biol. Chem.* 277, 13379 - 13388
39. Kirsch, M., Korth, H.-G., Sustmann, R., and de Groot, H. (2002): The pathobiochemistry of nitrogen dioxide. *Biol. Chem.* 383, 389 - 399
40. Kirsch, M., Lomonosova, E. E., Korth, H. G., Sustmann, R., and de Groot, H. (1998): Hydrogen peroxide formation by reaction of peroxynitrite with HEPES and related tertiary amines. Implications for a general mechanism. *J. Biol. Chem.* 273, 12716 - 12724
41. Kissner, R., and Koppenol, W. H. (2002): Product distribution of peroxynitrite decay as a function of pH, temperature, and concentration. *J. Am. Chem. Soc.* 124, 234 - 239
42. Korth, H.-G., Sustmann, R., Lommes, P., Paul, T., Ernst, A., de Groot, H., Hughes, L., and Ingold, K. U. (1994): Nitric oxide cheletropic traps (NOCTs) with improved thermal stability and water solubility. *J. Am. Chem. Soc.* 116, 2767 - 2777
43. Kuhn, D. M., and Geddes, T. J. (2002): Reduced nicotinamide nucleotides prevent nitration of tyrosine hydroxylase by peroxynitrite. *Brain Res.* 933, 85-89
44. Kwon, N. S., Nathan, C. F., and Stuehr, D. J. (1989): Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.* 264, 20496-20501

45. Lehnig, M. (1999): Radical mechanisms of the decomposition of peroxynitrite and the peroxynitrite-CO₂ adduct and of reactions with L-tyrosine and related compounds as studied by N-15 CIDNP. *Arch. Biochem. Biophys.* 368
46. Lohse, M. J., Förstermann, U., and Schmidt, H. H. H. W. (1998): Pharmacology of NO: cGMP signal transduction. *N-S Arch. Pharmacol.* 358, 111 - 112
47. Lomonosova, E., Kirsch, M., Rauen, U., and de Groot, H. (1998): The critical role of HEPES in SIN-1 cytotoxicity, peroxynitrite versus hydrogen peroxide. *Free Radic. Biol. Med.* 24, 522 - 552
48. Manzoni, O., Prezeau, L., Marin, P., Deshager, S., Bockaert, J., and Fagni, L. (1992): Nitric oxide induced blockade of NMDA receptors. *Neuron* 8, 653-662
49. Mastrocola, T., Lambert, I. H., Kramhoft, B., Rugolo, M., and Hoffmann, E. K. (1993): Volume regulation in human fibroblasts: role of Ca²⁺ and 5-lipoxygenase products in the activation of the Cl⁻ efflux. *J. Membr. Biol.* 136, 55 - 62
50. Matusa, K., and Imashiro, Y. (1970): *Chem. Pharm. Bull.* 18, 128
51. Mayer, B., Pfeiffer, S., Schrammel, A., Koesling, D., Schmidt, K., and Brunner, F. (1998): A new pathway of nitric oxide/cyclic GMP signaling involving S-nitrosoglutathione. *J. Biol. Chem.* 273, 3264-3270
52. Meineke, P., Rauen, U., de Groot, H., Korth, H.-G., and Sustmann, R. (1999): Cheletropic traps for the fluorescence spectroscopic detection of nitric oxide (nitrogen monoxide) in biological systems. *Chem. Eur. J.* 5, 1738-1747
53. Meineke, P., Rauen, U., de Groot, H., Korth, H.-G., and Sustmann, R. (2000): Nitric oxide detection and visualization in biological systems. Applications of the FNOCT method. *Biol. Chem.* 381, 575-582
54. Meinertz, T., Brandstatter, A., Trenk, D., Jahnchen, E., Ostrowski, J., and Gartner, W. (1985): Relationship between pharmacokinetics and pharmacodynamics of molsidomine and its metabolites in humans. *Am. Heart. J.* 109, 644-648

55. Minami, M., and Yoshikawa, H. (1979): A simplified assay method of superoxide dismutase activity for clinical use. *Clin. Chim. Acta* 92, 337-342
56. Oyanagui, Y. (1984): Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Anal. Biochem.* 142, 290-296
57. Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987): *Nature London* 327, 524
58. Petrat, F., Pindiur, S., Kirsch, M., and de Groot, H. (2003): NAD(P)H, a primary target of $^1\text{O}_2$ in mitochondria of intact cells. *J. Biol. Chem.* 278, 3298-3307
59. Petrat, F., Pindiur, S., Kirsch, M., and de Groot, H. (2003): "Mitochondrial" photochemical drugs do not release toxic amounts of $^1\text{O}_2$ within the mitochondrial matrix space. *Arch. Biochem. Biophys.* 412, 207 - 215
60. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991): Peroxynitrite oxidation of sulfhydryls: The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* 266, 4244-4250
61. Radi, R., Cassina, A., Hodara, R., Quijano, C., and Castro, L. (2002): Peroxynitrite reactions and formation in mitochondria. *Free Radic. Biol. Med.* 33, 1451-1464
62. Radi, R., Rodriguez, M., Castro, L., and Telleri, R. (1994): Inhibition of mitochondrial electron transport by peroxynitrite. *Arch. Biochem. Biophys.* 308, 89 - 95
63. Räthel, T. R., and Dirsch, V. M. (2003): Application of 4,5-diaminofluorescein to reliably measure nitric oxide released from endothelial cells in vitro. *Biol. Proced. Online* 5, 136-142
64. Ross, A. B., Mallard, W. G., Helman, W. P., Buxton, G. V., Huie, R. E., and Neta, P. (1998) NDRL/NIST Solution Kinetics Database 3.0. In *NDRL/NIST*, Gaithersburg, MD
65. Schmidt, H. H. H. W., Hofmann, H., Schindler, U., Shutenko, Z. S., Cunningham, D. D., and Feelisch, M. (1996): No NO from NO synthase. *Proc. Natl. Acad. Sci. USA* 93, 14492-14497
66. Schmidt, K., Pfeiffer, S., and Mayer, B. (1998): Reaction of peroxynitrite with HEPES or MOPS results in formation of nitric oxide donors. *Free Radic. Biol. Med.* 24, 859 - 862

67. Schönafinger, K. (1999): Heterocyclic NO prodrugs. *Il Farmaco* 54, 316 - 320
68. Schrammel, A., Pfeiffer, S., Schmidt, K., Koesling, D., and Mayer, B. (1998): Activation of soluble guanyl cyclase by the nitrovasodilator 3-morpholinosydnonimine involves formation of S-nitrosoglutathione. *Mol. Pharmacol.* 54, 207 - 212
69. Schweizer, M., and Richter, C. (1994): Nitric oxide potently and reversibly deenergizes mitochondria at low oxygen tension. *Biochem Biophys Res Commun* 204, 169-175
70. Sies, H., Sharov, V. S., Klotz, L.-O., and Briviba, K. (1997): Glutathione peroxidase protects against peroxynitrite-mediated oxidations. *J. Biol. Chem.* 272, 27812-27817
71. Singlas, E., and Martre, H. (1983): Human pharmacokinetics of molsidomine. *Ann. Cardiol. Angeiol.* 32, 503-509
72. Thome, U., Lazrak, A., Chen, L., Kirk, M. C., Thomas, M. J., Forman, H. J., and Matalon, S. (2003): Novel SIN-1 reactive intermediates modulate chloride secretion across murine airway cells. *Free Radic. Biol. Med.* 35, 662 - 675
73. Tsukahara, H., Gordienko, D. V., and Gologorsky, M. S. (1993): Continuous monitoring of nitric oxide release from human umbilical vein endothelial cells. *Biochem. Biophys. Res. Commun.* 193, 722 - 729
74. Tu, B., Wallin, A., Moldeus, P., and Cotgreave, I. (1995): The cytoprotective roles of ascorbate and glutathione against nitrogen dioxide toxicity in human endothelial cells. *Toxicology* 98, 125 - 136
75. Tyler, D. D. (1992): *The Mitochondrion in Health and Disease*, VCH Publishers, New York
76. Werringloer, J., Wolf, J., and Gans, G. (1990): Concomitant release of nitric oxide and generation of superoxide during non-enzymatic decay of SIN-1. *Eur. J. Pharmacol.* 183, 1616
77. Wilson, I. D., Fromson, J. M., and Illing, H. P. A. (1987): The metabolism of ¹⁴C N-ethoxycarbonyl-3-morpholinosydnonimine (molsidomine) in man. *Xenobiotica* 17, 93 - 104

78. Wilson, I. D., Watson, K. V., Troke, J., Illing, H. P. A., and Fromson, J. M. (1986): The metabolism of ^{14}C N-ethoxycarbonyl-3-morpholinosydnonimine (molsidomine) in laboratory animals. *Xenobiotica* 16, 1117 - 1128

9 APPENDIX: Abbreviations

ACE	angiotensine converting enzyme
BAEC	Bovine aortic endothelial cells
cGMP	cyclic guanosine monophosphate
$\text{CO}_3^{\bullet-}$	trioxocarbonate(1-)
DAF-2 DA	4,5-diaminofluorescein diacetate
FNOCT-1	Fluorescent Nitric Oxide Cheletropic Trap-1
MEM	minimum essential medium
MOPS	3-morpholino-propane-sulfone acid
NO	nitric oxide
NOS I-III	nitric oxide synthase I-III
ONOO^-	oxoperoxonitrate(1-)
$\text{ONOO}^-/\text{ONOOH}$	Peroxynitrite
ONOOH	hydrogen oxoperoxonitrate(1-)
PBS	phosphate buffered saline
RAS	renin angiotensin system
sGC	soluble guanylyl cyclase
SIN-1	3-morpholino-sydnonimine
SIN-1C	N-morpholinoiminosydnonimine
SOD	superoxide dismutase
spermineNONOate	(Z)-1- β -N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino β -diazene-1-ium-1,2diolate

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